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PI - Signature Date

Cold Spring Harbor Laboratory
Conference On
MICROBIAL PATHOGENESIS AND HOST REPSPONSE

September 22 – 26, 1999

268 participants

ARRANGED BY: **P.T. Magee,** ***University of Minnesota***
Stanley Maloy, ***University of Illinois***
Ronald Taylor, ***Dartmouth Medical School***

Understanding microbial pathogenesis demands a detailed knowledge of the host response as well as the pathogen itself, and requires an interdisciplinary approach, integrating the fields of microbiology, eukaryotic cell biology, and immunology. The Cold Spring Harbor meeting on Microbial Pathogenesis and Host Response was planned to facilitate such interactions. The meeting attracted 268 international scientists who approach the study of bacterial and fungal pathogens from a broad range of perspectives. The meeting included 57 talks and 140 posters. Each session focused on a specific mechanism of virulence in bacteria and fungi. Each session was littered with significant new insights into the microbial-host interaction. A few highlights of these sessions are described below.

The first session dealt with adhesion of microbial pathogens to the eukaryotic host. Paula Sundstrom (Ohio State University) presented evidence that adhesion of Candida albicans to epithelial cells involves covalent attachment via cross-linking of cell surface ligands by mammalian transglutaminases. Using C. albicans mutants deficient for this interaction, she demonstrated that the covalent attachment is essential for colonization and subsequent virulence in mice. Several talks discussed the role of fimbriae in adhesion of bacterial pathogens. Rob Edwards (University of Illinois) presented the first evidence that fimbriae may mediate adhesion to tissues within the host in addition to host surfaces such as the cells lining the intestine. Andreas Baumler (Texas A&M) and Marian van der Woude (University of Pennsylvania) discussed the role of fimbrial phase variation in eluding host defense mechanisms.

Several sessions focused on how microbial pathogens sense different microenvironments in the host and regulate gene expression in response to these environments. C. Lee (Harvard University) presented an overview of the diversity of signals required to control a cascade of gene expression during Salmonella infections. Several talks described clever new genetic tools for identifying potential virulence genes. Subsequent talks described the regulatory mechanisms that respond to host signals in other bacteria and fungi. Hans Wolf-Watz (Umea University, Sweden), Jorge Galan (Yale University), Jose Puente (UNAM, Mexico), and Kim Orth (University of Michigan) described how type III secretion systems allow bacteria to inject proteins into eukaryotic cells and how these proteins enslave the eukaryotic host by modulating the signal transduction pathways and cytoskeletal rearrangements.

Additional sessions focused on host defenses to microbial pathogens. Brad Cookson (Universtiy of Washington) gave an excellent overview of host defense mechanisms and described his own research on how T-cells recognize specific epitopes of bacterial pathogens. Ferric Fang (University of Colorado) described the role on oxygen radicals and nitrous oxide in the initial attack on microbial invaders. J. Goguen (University of Massachusetts) described the use of mutant strains of mice have been used to study the role of plasminogen in resistance to Yersinia pestis infection, the causative agent of plague. Other talks described host responses to a battery of different bacteria and fungi.

One session focused on what we have learned about microbial virulence and the evolution of virulence from genome analysis. George Weinstock (University of Texas Health Sciences Center) discussed the genomic analysis of pathogenic spirochetes. The complete genome sequence of Treponema pallidum, the causative agent of syphilis, uncovered previously obscured insights into the physiology of this pathogen that is so persnickety it cannot be cultured in vitro. B. B. Magee (Univ. Minnesota) discussed the current status of the Candida albicans genome sequence. The genome analysis facilitated identification of a novel mechanism of karyotypic variation in C. albicans.

One of the surprises from the microbial genome sequencing projects was the extent of lateral gene transfer between different microbes, as indicated by islands of DNA with a different GC content and/or codon preference than the rest of the genome. Many of these islands contain sequences similar to known phage genes. In the pre-genomic era the bacterial chromosome was thought to be a graveyard for "dead" phage. Three talks emphasized that in contrast to this pre-genomic perspective, "bacteria act as hotels for phage that may be sleeping but are very much alive" and play important roles in the mechanism and evolution of bacterial virulence. Matthew Waldor (Tufts University) described the role of filamentous phage in transfer of cholera toxin between Vibrio cholerae strains, and the role of a lambdoid phage in expression of the shiga-like toxin (Stx) in E. coli O157:H7. This strain of E. coli has caused serious outbreaks of food related illnesses, most notably the "Jack-in-the-Box" outbreak in Washington State. The Stx toxin is responsible for severe clinical sequelae, such as the hemolytic uremic syndrome responsible for many deaths. Expression of Stx is specifically induced by the phage late genes, thus induction of lytic growth of the phage enhances virulence of E. coli O157:H7. This leads to a practical paradox: because the phage itself produces the toxin, treatment of infected patients with antibiotics that kill the bacteria and induce the phage can acerbate the clinical symptoms. Lionello Bossi (CNRS, Gif-sur-Yvette, France) described two lysogenic phage on the chromosome of Salmonella Typhimurium. These phage transfer numerous virulence genes between Salmonella serovars. T. Ho (Univ. Illinois) described a gene located on Gifsy-2 that has an avirulence phenotype-that is, mutations in this gene increase virulence relative to the wild-type strain. This is the first true avirulence gene described in Salmonella, and emphasizes the unique role of the phage in modulating bacterial virulence. Together these three talks emphasized the important role of phage in the simultaneous acquisition of multiple virulence determinants, and showed that the phage are fully active and able to lyse and lysogenize their hosts under suitable conditions.

In summary, this session conveyed a variety of exciting new insights into the understanding of how microbial virulence mechanisms evolve, and suggested that with appropriate perspicacity genomic analysis can provide clues to virulence determinants and their acquisition. The progress in this front has resulted from an orgy of intellectual exchange between the fields of genomics, genetics, biochemistry, and cell biology. The availability of genome sequences is beginning to provide glimpses the evolution of microbial virulence. Furthermore, the number of completed genome sequences is increasing rapidly due to the confluence of cheaper techniques and the accumulation of sequencing power. Thus, it seems clear that over the next few years the accretion of additional genome sequences will provide comparative insights into how virulence genes are acquired and how they open new physiological niches for bacterial pathogens. The insights from these studies are likely to lead to practical applications in disease management and treatment that would have been inconceivable less than a decade ago.

The final oral session focused on emerging pathogens and novel environmental niches. Patti Fields (Centers for Disease Control) gave an overview of the growing impact of emerging pathogens. Howard Shuman (Columbia University, NY, USA) summarized the work of a number of labs on the origin and function of genes from Legionella pneumophilia that are required for intracellular multiplication within both human macrophages and free-living protozoa. The cluster of Legionella genes required for intracellular growth are not required for growth in vitro and appear to encode a type IV secretion

apparatus which transfers effector molecules into host cells. Based upon genetic and genomic comparisons with related bacteria, acquisition of these genes is likely to have been a giant step for Leigonella to make the evolutionary leap to an intracellular niche. Jim Russell (Cornell University) described how modern feeding methods contribute to the dissemination of pathogenic E. coli from cattle to humans. Kali Mathee (Flordia International Univ.) and George O'Toole (Cornell Univ.) described novel approaches for studying biofilm formation by Pseudomonas aeruginosa, a serious opportunistic pathogen for patients with cystic fibrosis.

Additional talks described clever new model systems for dissecting virulence mechanisms in other bacteria.

In addition to the oral sessions, the poster sessions presented a variety of new insights into bacterial and fungal pathogens and host defenses to microbial pathogens. Both the oral and poster sessions stimulated lively interactions between the participants.

Finally, Stanley Falkow (Stanford University) gave an inspiring, introspective talk on future challenges facing the field of microbial pathogenesis. He discussed the importance of the numerous pathogens that have not yet been adequately studied due to the lack of suitable molecular tools, and argued that the application of new tools may make these organisms more pliable. As an example, he described how his lab is using DNA-chips to identify potential virulence determinants in Helicobacter pylori, a major cause of stomach ulcers and cancer.

Reviews of this meeting are in press in Trends in Microbiology and Trends in Genetics. Based upon the support and enthusiasm of the participants, the meeting was a resounding success. Thus, a second Microbial Pathogenesis and Host Response meeting will be held in Fall 2001.

This meeting was funded in part by the National Institute of Allergy and Infectious Diseases, the United States Army, AstraZeneca R and D Boston, Inc, Burroughs Wellcome, Intrabiotics Pharmaceuticals, Inc, and Pharmacia & Upjohn Inc. Contributions from the Corporate Sponsors and Plant Corporate Associates also provided core support for this meeting.

PROGRAM

WEDNESDAY, September 22—7:30 PM

INTRODUCTION

Stanley Maloy

SESSION 1 ADHESION TO THE HOST

Chairperson: **P. Orlean,** University of Illinois, Urbana-Champaign

THURSDAY, September 23—9:00 AM

SESSION 2 REGULATORY RESPONSES TO ENVIRONMENTAL CUES

Chairperson: **C. Lee,** Harvard Medical School, Boston, Massachusetts

THURSDAY, September 23—2:00 PM

SESSION 3 POSTER SESSION I

THURSDAY, September 23—7:00 PM

SESSION 4 VIRULENCE DETERMINANTS THAT MANIPULATE THE HOST REPONSE

Chairperson: **R. Taylor,** Dartmouth Medical School, Hanover, New Hampshire

FRIDAY, September 24—9:00 AM

SESSION 5 SURVIVAL IN THE HOST

Chairperson: **S. Libby,** North Carolina State University, Raleigh

FRIDAY, September 24—4:00 PM

SESSION 6 POSTER SESSION II

FRIDAY, September 24—7:00 PM

SESSION 7 HOST DEFENSES

Chairperson: **B. Cookson,** University of Washington, Seattle

SATURDAY, September 25—9:00 AM

SESSION 8 EVOLUTION OF PATHOGENS

Chairperson: **S. Maloy,** University of Illinois, Urbana

SATURDAY, September 25—1:30 PM

SESSION 9 TOXINS AND SUPERANTIGENS

Chairperson: **J. Scott,** Emory University, Atlanta, Georgia

SATURDAY, September 25—4:30 PM

SESSION 10 FUTURE PERSPECTIVES

Keynote Speaker

Stanley Falkow
Stanford University, California
"The pathogenesis of *S. typhimurium* infection"

SUNDAY, September 26—9:00 AM

SESSION 11 EMERGING PATHOGENS, ENVIRONMENTAL NICHES, AND MODEL SYSTEMS

Chairperson: **P. Fields,** Centers for Disease Control and Prevention, Atlanta, Georgia

Abstracts of papers presented
at the 1999 meeting on

MICROBIAL PATHOGENESIS & HOST RESPONSE

September 22–September 26, 1999

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

This meeting was funded in part by the **National Institute of Allergy and Infectious Diseases,** a branch of the **National Institutes of Health;** the **United States Army; AstraZeneca R and D Boston, Infection; Burroughs Wellcome; Intrabiotics Pharmaceuticals, Inc.;** and **Pharmacia & Upjohn Inc.**

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Albert B. Sabin Vaccine Institute, Inc. at Georgetown University

Cover: GFP-labeled *Pseudomonas aeruginosa* forming microcolonies on a monolayer of cells in vitro. Courtesy of George O'Toole, Jr, Dept. of Microbiology, Dartmouth Medical School.

MICROBIAL PATHOGENESIS & HOST RESPONSE
Wednesday, September 22 - Sunday, September 26, 1999

Wednesday	7:30 pm	**1** Adhesion to the Host
Thursday	9:00 am	**2** Regulatory Responses to Environmental Cues
Thursday	2:00 pm	**3** Poster Session I
Thursday	4:30 pm	Wine and Cheese Party *
Thursday	7:00 pm	**4** Virulence Determinants that Manipulate the Host Response
Friday	9:00 am	**5** Survival in the Host
Friday	4:00 pm	**6** Poster Session II
Friday	7:00 pm	**7** Host Defenses
Saturday	9:00 am	**8** Evolution of Pathogens
Saturday	1:30 pm	**9** Toxins and Superantigens
Saturday	4:30 pm	**10** Future Perspectives-**Keynote Address**
Saturday	7:00 pm	Banquet
Sunday	9:00 am	**11** Emerging Pathogens, Environmental Niches, and Model Systems

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Poster sessions are located in *Bush Lecture Hall*
* *Airslie Lawn*, weather permitting
Mealtimes at Blackford Hall are as follows: Breakfast 7:30am-9:00am
Lunch 11:30am-1:30pm
Dinner 5:30pm-7:00pm
Bar times are from 5:00pm until late

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"The pathogenesis of *S. typhimurium* infection" 201

SATURDAY, September 25

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

Following banquet—Entertainment provided by
The Jeff Brown Band

SUNDAY, September 26—9:00 AM

SESSION 11 EMERGING PATHOGENS, ENVIRONMENTAL NICHES, AND MODEL SYSTEMS

Chairperson: **P. Fields,** Centers for Disease Control and Prevention, Atlanta, Georgia

Russell, J.B., Diez-Gonzalez, F., Jarvis, G.N., Agricultural Research Service, USDA, Dept. of Microbiology, Cornell University, Ithaca, New York: The effect of grain-feeding on the dissemination of *E. coli* from cattle to man. 202

O'Toole, G.A.,[1] Kolter, R.,[2] [1]Dartmouth Medical School, Hanover, New Hampshire; [2]Harvard Medical School, Boston, Massachusetts: A role for biofilm development in *P. aeruginosa* pathogenesis. 203

AUTHOR INDEX

THE ROLE OF THE *CANDIDA ALBICANS* ADHESIN HYPHAL WALL PROTEIN 1 IN CANDIDIASIS.

Paula Sundstrom, The Ohio State University.

The indigenous commensal *Candida albicans* is emerging as a major human opportunistic pathogen because of increasing numbers of immunosuppressed individuals. Progression from commensalism to mucosal or systemic candidiasis involves invasion of keratinized epithelial cells by hyphae. Loss of innate and specific immune host defenses is primarily responsible for susceptibility to opportunistic candidiasis arising from endogenous *C. albicans* in the normal flora. However, pro-adhesive and pro-invasive factors of *C. albicans* also contribute to disease by mediating proliferation and penetration of host tissues when risk factors are present.

The *HWP1* gene is expressed in germ tubes and true hyphae and encodes a surface protein, Hwp1. Hwp1 is predicted to be an outer mannoprotein with a cell surface-exposed, ligand-binding domain at the N-terminus and C-terminal features that confer covalent integration into the β-glucan of the cell wall. The N-terminal domain of Hwp1 serves as a substrate in cross-linking reactions mediated by mammalian transglutaminases (TGases). Hwp1 is involved in transglutaminase-mediated cross-linking of *C. albicans* to BEC's. Stabilized adhesion of the homozygous *hwp1/hwp1* mutant strain is reduced by 75% compared to strains expressing at least one intact *HWP1* gene. The stability of germtube:BEC complexes observed in vitro is typical of that found in patient samples of pseudomembranous candidiasis.

Direct evidence for a role of *HWP1* in candidiasis was studied in murine models of mucosal and systemic candidiasis. Oral colonization of immunodeficient mice with the homozygous *hwp1/hwp1* mutant did not cause extensive illness and death that was found in mice colonized with isogenic strains harboring *HWP1*. The importance of *HWP1* in systemic candidiasis was supported by an increase in survival in mice intravenously injected with the homozygous *hwp1/hwp1* mutant compared to the other strains. These findings suggest that inhibition of expression of Hwp1 on hyphal surfaces or interference with transglutaminase-mediated cross-linking will provide new strategies for therapeutic intervention in candidiasis.

HOW DOES *SALMONELLA* BENEFIT FROM FIMBRIAL PHASE VARIATION ?

Andreas J. Bäumler, Department of Medical Microbiology and Immunology, Texas A&M University Health Science Center, College Station, TX

Conventional wisdom holds that phase variation is a mechanism for immune evasion. However, despite fimbrial phase variation, mice previously exposed to *Salmonella typhimurium* are protected against a subsequent challenge. We evaluated whether *lpf* phase variation may instead be a mechanism to evade cross immunity between *Salmonella* serotypes. Mice were immunized orally with *S. typhimurium aroA* mutants which either expressed the *lpf* operon (phase ON variant) or in which the entire *lpf* operon had been removed by deletion. During a subsequent challenge with virulent *Salmonella enteritidis* a selection against *lpf* phase ON variants was observed in mice previously exposed to *S. typhimurium* LP fimbriae. Vaccination with *S. typhimurium* did not confer protection against challenge with *S. enteritidis*, presumably because *lpf* phase OFF variants were able to evade cross-immunity. We propose that *lpf* phase variation is a mechanism to evade cross-immunity between *Salmonella* serotypes, thereby allowing their coexistence in a host population.

Specific Interactions between *S. enteritidis* and macrophages.

Robert Edwards, Dieter Schifferli and Stanley Maloy. University of Illinois at Urbana Champaign, Urbana, IL 61801

Unlike other fimbriae which are involved in attachment to epithelial surfaces, the *Salmonella enteritidis* SEF fimbriae are specifically required for resistance to macrophage attack in the peritoneum. Polar *sef* mutants which abolish downstream gene expression cannot cause systemic infection of mice. Mutations which only abolished the major structural subunit had no phenotype. Adhesin mutants were avirulent and unsuccessful at macrophage internalization in the peritoneum. Using fluorescent-labeled bacteria and flow cytometry we have examined the interaction between *S. enteritidis* and macrophages and suggest a novel role for fimbriae in systemic infections.

DNA METHYLATION-DEPENDENT PHASE VARIATION OF THE OUTER MEMBRANE PROTEIN AG43 IN E. COLI.

Marjan van der Woude, Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6076.

Regulating the expression of virulence factors is an important factor in bacterial pathogenesis. The expression of a group of fimbrial adhesins in *Escherichia coli*, including Pap, is under the control of a phase variation mechanism which requires DNA methylation by deoxyadenosine methylase (Dam). Regulation by DNA methylation patterns allows the transcriptional state of *pap* to be heritable but reversible. The biological role or extent of this type of methylation-dependent, epigenetic regulation is not known. Indeed, phase variation of the *pap*-like family of fimbriae is the only well characterized Dam-dependent regulatory system. However, Heithoff et al recently showed that Dam is required for virulence of *Salmonella typhimurium*, suggesting Dam may be a global regulator. In order to understand the significance of methylation-dependent regulation, it is important to identify and characterize other Dam-dependent systems. We are analyzing phase variation of the outer membrane protein Ag43 in *E. coli*. The biological role of Ag43 has not been elucidated but there is homology to the adhesin AIDA-I and expression leads to bacterial auto-aggregation, suggesting a role in bacterial adhesion. Phase variation of Ag43, encoded by *agn*, requires Dam and OxyR, a global regulator of the oxidative stress response. We show that transcription of *agn* is repressed by OxyR and activation requires Dam. Other members of the OxyR regulon are differentially regulated by the reduced versus oxidized state of this protein. In vivo, reduced OxyR (OxyR[C199S] mutant) was sufficient to repress Ag43 transcription. However, in vitro both oxidized and reduced forms of OxyR bound to the regulatory region and binding of both forms was decreased by Dam-dependent methylation of three GATC sequences in its binding site. It remains to be determined if oxidized OxyR can repress transcription. In addition, in vivo, the methylation state of the *agn* DNA correlated to the transcription state, and methylation protection required OxyR. These results are consistent with a model in which methylation serves to abrogate OxyR binding at the *agn* regulatory region, thereby relieving repression. However, even in the absence of OxyR Dam is required for full activation, suggesting an additional, as yet unidentified role for methylation. Analyses of Pap and Ag43 expression shows that Dam-dependent regulation overlaps with two regulons (Lrp and OxyR, respectively), supporting a hypothesis that Dam is a global regulator in addition to its well recognized role in various housekeeping functions.

ULTRASTRUCTURAL ANALYSIS OF CLOSTRIDIAL ENDOSPORE ATTACHMENTS TO HUMAN CELLS

Barbara Panessa-Warren, George T. Tortora, SUNY at Stony Brook, NY,
John B. Warren, Brookhaven National Laboratory, Upton, NY

The endospores of *C. difficile* ATCC 9689 and 43594 and *C. sporogenes* ATCC 3584 demonstrate morphological changes in the exosporial membrane (outer covering of the spore) that facilitate attachment. Prior to spore activation, the exosporial membrane is smooth and closely adheres to the underlying spore coat. At activation the exosporium differentiates, producing appendages that extend to a nutritive surface such as agar, or to host cells. This study analyzes clostridial spore differentiation, attachment and host cell response by scanning, transmission (TEM), and Field emission (FEM) electron microscopy, atomic force microscopy (AFM), and light microscopy. *C. difficile* 43594 (an isolate from a patient with pseudomembranous colitis) when anaerobically incubated with human embryonic fibroblast monolayers (MRC-5 cells) did not attach. Yet when these spores were similarly incubated with human colon carcinoma confluent monolayers (Caco-2 cells) the exosporia developed ruthenium red positive (+) structures that by TEM were found to fuse with the host cell microvillar membranes. FEM revealed that the endospores only attached to host cells with microvilli, and that numerous spores attached to each cell. Thin sections revealed the entry of the spore protoplast (no gram positive cell wall) into the host cell cytoplasm, with the exosporia and spore coats left outside on the microvillous surface. The protoplast was seen in the cytoplasm without a vacuolar membrane. In contrast, *C. difficile* 9689(a standard laboratory QC strain), when anaerobically incubated with Caco-2 cells did not enter the host cell. But the exosporium did attach to microvilli and the cell surface, where the spores germinated, eventually causing the digestion of the host cell cytoplasm ground substance. FEM of *C. difficile* 9689 activated endospores showed extensive clavate exosporial appendages, which when thin sectioned appeared ruthenium red +; the latter areas form the attachment to the host cell membrane. *C. sporogenes* endospores upon activation showed myriads of delicate long and short filamentous exosporial ruthenium + extensions which attached the spore to agar or to host cells. When anaerobically incubated with MRC-5 fibroblast monolayers, the clostridial exosporium directly attached to the host cell cytoplasm by the ruthenium + extensions. This attachment was subsequently replaced, following focal digestion of the fibroblast plasmalemma, by a more substantial attachment of the exosporium proper into the host cell cytoplasm. By TEM at late germination the exosporium anchored directly into the cytoplasm, allowing the spore to complete germination. FEM revealed outgrowth and colonization of the newly formed vegetative cells onto the fibroblast surface. AFM has shown that clostridial exosporial attachment structures on living spores were not deformed or removed by the action of the probe repeatedly moving across the exosporial surface.

IDENTIFICATION OF A HEPARIN-BINDING HEMAGGLUTININ OF *BORRELIA BURGDORFERI* AS A CANDIDATE GLYCOSAMINOGLYCAN-BINDING ADHESIN

Parveen, Nikhat and J. M. Leong
Deptartment of Molecular Genetics and Microbiology
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Lyme disease, caused by the tick-borne spirochete *Borrelia burgdorferi*, has emerged in the past two decades to become the most common arthropod-borne illness in the U.S. If untreated, the infection may result in a chronic illness that can affect the skin, joints, heart and nervous system. The interaction of this bacterium with host cells is likely to be critical in the chronic infection of multiple tissues. Glycosaminoglycans (GAGs), which are long, linear, highly sulfated carbohydrates present on the host cell surface, have been shown to mediate attachment of the spirochetes to mammalian cells in vitro. Although different *B. burgdorferi* strains show different GAG-binding preferences, most strains recognize heparin/heparan sulfate and dermatan sulfate but not chondroitin sulfate. The ability to bind to GAGs is associated with a heparin-inhibitable hemagglutination activity.

In this study, we have identified a *B. burgdorferi* heparin-binding hemagglutinin that is a strong candidate for a GAG-binding adhesin. We first identified five *B. burgdorferi* proteins that bound both to hemagglutination-competent erythrocytes and to heparin-agarose beads. The presence of one of these proteins, a species with an apparent molecular weight of 26 kD, correlated best with hemagglutination activity. N-terminal sequencing of this protein revealed that it corresponds to a 241 amino acid protein with a cleaved signal sequence. We have termed this protein Bgp (*Borrelia* GAG-binding protein). An 18 amino acid sequence of Bgp shows features similar to those of the heparin binding domains of two well-characterized GAG-binding proteins, heparin cofactor II and anti thrombin III. Three independent approaches suggested that Bgp protein is associated with outer membrane of the spirochete. First, extracts were generated from intact spirochetes that had been treated with a membrane-impermeant biotinylating agent, and among the extract proteins were adsorbed to erythrocytes or heparin sepharose, a 26 kD erythrocyte-binding species (presumably Bgp) was found to be biotinylated. Second, anti-Bgp antiserum raised in mice recognized a 26 kD band present in purified outer membrane vesicles of *B. burgdorferi*. Finally, anti-Bgp antiserum recognized intact spirochetes as determined by indirect immunofluoresence. Finally, recombinant Bgp agglutinated erythrocytes and bound to GAGs with the identical GAG-binding preference as the parental *B. burgdorferi* strain. Recombinant Bgp also agglutinated erythrocytes. The localization of Bgp to the outer membrane and the hemagglutination and GAG-binding properties of this protein indicate that Bgp is a likely candidate for a GAG-binding adhesin of *B. burgdorferi*.

HOW BACTERIA TALK TO EACH OTHER: QUORUM SENSING IN *ESCHERICHIA COLI*, *SALMONELLA TYPHIMURIUM* AND *VIBRIO HARVEYI*

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In bacteria, the regulation of gene expression in response to changes in cell population density is called quorum sensing. Quorum sensing bacteria produce, release and respond to extracellular signalling molecules called autoinducers that accumulate in the environment as the cell population increases. In the luminous marine bacterium *V. harveyi*, two independent quorum sensing systems exist, and each is composed of a sensor-autoinducer pair. Signalling from both sensory systems converges at a shared integrator protein that controls the expression of the luciferase structural operon (*luxCDABE*). The mechanism of signal relay is a complex two-component phosphorylation/dephosphorylation cascade. Our results suggest that *V. harveyi* uses System 1 for intra-species communication and System 2 for inter-species cell-cell signalling. We determined that many species of bacteria, including *Escherichia coli* O157 and *Salmonella typhimurium* 14028, produce autoinducers similar or identical to the *V. harveyi* System 2 autoinducer (AI-2). However, unlike in other described quorum sensing systems in which autoinducer accumulates in stationary phase, in *E. coli* and *S. typhimurium* maximal autoinducer activity is produced during mid-exponential phase and the signal is degraded by the onset of stationary phase. Protein synthesis is required for degradation of the activity, suggesting that a complex regulatory circuit controls signal production, detection and response in *E. coli* and *S. typhimurium*. We have identified and cloned the gene responsible for AI-2 production from *V. harveyi*, *S. typhimurium* and *E. coli*, and we call the locus *luxS*. The *luxS* genes are highly homologous and define a new family of autoinducer production loci that are widespread among both Gram negative and Gram positive bacteria.

EFFECT OF ENVIRONMENTAL pH ON MORPHOLOGICAL DEVELOPMENT AND VIRULENCE OF *CANDIDA ALBICANS* .

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Candida albicans is regarded as a commensal of the gut with no known external reservoirs. It survives not only within this environment, but can infect a broad range of host niches including most mucosal surfaces, skin, and internal organs. Infection of these diverse environments undoubtedly depends on the organism's ability to cope with the distinctive stresses imposed by each. As bacterial pathogens rely upon environmental parameters within the host milieu to signal the expression of critical virulence determinants, the effect of these parameters on gene expression in *C. albicans* is being investigated. Ambient pH has been show to modulate the expression of several genes in *C. albicans*. Two of these, *PHR1* and *PHR2*, encode functional homologs that are required for normal morphogenesis of both yeast and hyphae. *PHR1* is required at neutral to alkaline pH when *PHR2* is not expressed and, conversely, *PHR2* is required at acidic pH when *PHR1* is not expressed. Indirect evidence indicates a similar response *in vivo*, which is essential to a successful infection. A signaling pathway that appears to be conserved in pathogenic and nonpathogenic fungi controls expression of these genes. In *C. albicans* this pathway controls not only morphogenesis per se, but also controls functions specific to hyphal development, an attribute integral to the virulence of this organism. Thus, the response to ambient pH plays a critical role in the biology of candidal pathogenesis.

VST1, A SMALL NONCODING RNA INVOLVED IN CELL-CELL COMMUNICATION IN THE *USTILAGO MAYDIS* / MAIZE SYSTEM.

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The phytopathogenic fungus *Ustilago maydis* is a facultative pathogen and certain steps of its life cycle strictly depend on its maize host. *U.maydis* exists in two morphological stages. Haploid sporidia grow yeast like and are non pathogenic, while the fusion product of compatible sporidia, the filamentous dikaryon, is able to infect plants. Mating and filamentous growth are regulated by the two mating type loci *a* and *b*. The *a* locus encodes a pheromone/receptor system and controls cell fusion. After fusion, the regulatory products of the *b* locus are required for subsequent pathogenic development.

To tag differentially expressed genes which are expressed during different steps of the life cycle, we developed a REMI (restriction enzyme mediated insertion) based enhancer trap screening system using GFP as reporter gene.

Here we describe a mutant that shows strong *egfp* induction during mating, but not under any other condition. The insertion was found in a region where no ORF could be identified. However, in the vicinity of the original insertion a small transcript could be detected whose expression pattern parallels the *egfp* expression pattern of the original REMI mutant. This very short transcript of about 40 nucleotides (*vst1*) is not polyadenylated and does not contain an ORF. *vst1* null mutants are strongly attenuated in mating and show significantly reduced levels of pheromone gene transcripts. We also present evidence for a second transcript, *vst2*, which is related in sequence to *vst1*. Vst molecules are proposed to act as a regulatory RNA species which affect gene expression through a novel mechanism.

IDENTIFICATION OF *ESCHERICHIA COLI* K1 GENES REQUIRED FOR VIRULENCE IN A SYSTEMIC MODEL OF INFECTION BY SIGNATURE-TAGGED MUTAGENESIS

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Strains of *E.coli* that possess the K1 capsule are predominant amongst colonic *E.coli* in 15% of healthy individuals. However, these strains are also a major cause of neonatal meningitis and septicaemia. Signature-tagged transposon mutagenesis allows the large-scale identification of genes that are required for survival of microbes *in vivo*. In order to identifiy genes that are involved in the virulence of this Gram negative pathogen a library of 1900 signature-tagged transposon mutants was screened in a murine model of systemic infection. Forty transposon mutants were identified that appeared to be attenuated in virulence in this model. The attenuation of these 40 mutants was evaluated in mixed infections with the wild type. The DNA flanking the transposon insertion sites in these 40 mutants has been cloned and sequenced. Twelve of the mutants have insertions in genes within the capsule biosynthesis gene cluster, confirming the importance of the polysialic acid capsule for the systemic survival of this organism. Fourteen of the mutants have insertions in genes that are either involved in LPS biosynthesis, lead to auxotrophy or are known to be virulence factors in *E.coli* or other organisms. The remaining mutants have insertions in genes that are either of unknown function or have not previously been implicated in bacterial virulence.

PROBING FOR BACTERIAL *IN VIVO* INDUCED GENES IN HUMAN INFECTIONS.

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We describe the design and preliminary testing of a new strategy for studying microbial pathogenesis and host interactions. Like other *in vivo* expression technology (IVET)-like approaches, our method is designed to identify genes of a pathogen that are expressed only during an actual infectious process. The major conceptual and technological breakthrough is that our method does not rely on animal models. Instead, it identifies genes expressed during an actual human infection. *In Vivo* Induced Antigen Technology (IVIAT), as we call it, uses pooled sera from patients to probe for genes expressed exclusively *in vivo*. IVIAT was initially developed to study the oral pathogen, *Actinobacillus actinomyce-temcomitans* (Aa). Sera from localized juvenile periodontitis patients were pooled and repeatedly absorbed with *in vitro* grown cells and lysates, leaving antibodies against antigens expressed only *in vivo*. An expression library of Aa genomic DNA was generated in *Escherichia coli* and clones were probed with the absorbed sera. Reactive clones were purified and DNA inserts were sequenced. A total of 8 *in vivo* induced (IVI) genes have been identified to date. Several are homologous to previously reported virulence factors in Aa or in the closely related organism, *Haemophilus influenzae*. We also identified genes for proteins of unknown function, which may represent novel virulence factors. To verify that the IVI antigens are indeed expressed by Aa during an actual infectious process in human subjects, the recombinant proteins will be purified and specific antibodies against them will be used to probe biological samples taken directly from infected patients. In addition to eliminating the need for animal models, IVIAT has several other advantages compared to IVET-like strategies. Interestingly, to date, none of the ORFs identified by IVIAT have homology to genes with known housekeeping function. Also, the use of pooled sera allows us to study antigens produced in different stages of infection and from patients infected by different routes, thereby maximizing the spectrum of IVI proteins recovered. Additionally, genomic libraries of a pathogen can be made using DNA pooled from different strains and clonotypes, again to optimize the recovery of IVI proteins. Finally, any cultivable pathogen can be analyzed by IVIAT without the need for sophisticated genetic constructions. IVI antigens discovered using IVIAT are excellent candidates for therapeutic and diagnostic targets and for vaccine design.

REGULATION OF *SALMONELLA TYPHIMURIUM* INVASION GENES BY FERMENTATION ACIDS

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The normal flora of the human intestine produce organic acids as terminal products of anaerobic metabolism. These metabolites accumulate in the gut to considerable concentrations and thus may serve as a biochemical marker of anatomical location for other bacteria, *e.g.*, intestinal pathogens such as *Salmonella typhimurium* which are known to restrict expression of virulence genes to appropriate host environments. In addition, the extent to which organic acids accumulate within bacterial cells is driven by the pH gradient across the cell envelope and thus provides a potential mechanism whereby the pH of the milieu may be converted into a chemical signal within the bacterial cytoplasm. Organic acids diffuse into bacteria in their un-ionized (HA) form and then ionize in the cytoplasm, with the consequence that accumulation of organic acid is accompanied by acidification of the cytoplasm. To begin to assess the role of organic acids in regulating *Salmonella* virulence genes, we have studied the effect of salicylic acid (*o*-hydroxybenzoic acid), a non-metabolized model compound, on invasion gene expression. This compound strongly represses several different invasion gene - *lacZ* operon fusions (*invF, sipC, prgH, hilA)* to a degree which varies with the concentration of salicylate and is more pronounced the lower the medium pH, as predicted from a basic chemical equilibrium model of this process. This inhibition occurs in the presence or absence of known regulators of invasion gene expression, including *hilA*, *sirA*, and *barA*. We have isolated both spontaneous mutations and transposon insertions that confer salicylate-resistant expression of invasion genes. Both the inhibition by salicylate and the action of the mutations are specific to invasion genes, as random fusions to *lacZ* are indifferent to these manipulations. Interestingly, some of the spontaneous salicylate-resistant mutations are found in the *rpoB* gene, encoding the β subunit of RNA polymerase. These observations suggest that salicylate may be affecting a basic property of the transcription apparatus itself to regulate invasion gene expression. In general, our results suggest that fermentation acids are negative regulators of virulence and may have a role in limiting *Salmonella* colonization to regions where the normal flora are comparatively sparse, *i.e.,* ileum and early large intestine.

TOXR FUNCTIONS AS AN ENHANCER PROTEIN FOR TCPP-MEDIATED ACTIVATION OF THE *TOXT* PROMOTER

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Virulence gene expression in *V. cholerae* is controlled by a cascade of transcriptional activators. Under appropriate growth conditions the inner membrane proteins ToxR and TcpP cooperate to activate the promoter of the gene encoding a third regulatory protein, ToxT. ToxT subsequently activates a number of virulence gene promoters, including those for cholera toxin (CT) and the toxin co-regulated pilus (TCP). Footprinting experiments using *V. cholerae* membranes demonstrated that in the absence of ToxR, TcpP protects a region of the *toxT* promoter from -43 to -57 relative to the transcription start site. This puts TcpP in close proximity to the consensus RNA-polymerase binding site. ToxR protects a region of the promoter further upstream from -68 to -104. However, when both ToxR and TcpP are present only the ToxR binding site is protected from DNAse I digestion, suggesting that under conditions where *toxT* is activated, TcpP does not bind DNA. Accordingly, specific residues were mutated in TcpP that are predicted to affect DNA binding or transcriptional activation based on homologous regulators. Derivatives were tested for their ability to activate *toxT* based on measurement of CT and TcpA (pilin subunit) production. While overexpressed wild type TcpP results in intermediate CT levels in the absence of ToxR, two TcpP mutants, W68L and R86A, failed to activate virulence gene expression when overexpressed, even in the presence of ToxR. A third TcpP derivative, H93L, failed to activate virulence gene expression when overexpressed on its own. However, when H93L was co-expressed with ToxR the levels of both CT production and TcpA expression were restored to nearly wild type levels. This suggests that in the absence of ToxR this TcpP derivative cannot interact effectively with the *toxT* promoter, but ToxR can suppress this defect, presumably by providing the promoter recognition function. While ToxR directly activates some promoters (*ompU*), its role in *toxT* activation appears to be different in that TcpP is also required. Furthermore, a *V. cholerae* mutant harboring a ToxR derivative, G80S, has reduced levels of OmpU, while TcpA and CT levels in this strain are similar to those of wild type. Thus, the transcriptional activation capability of ToxR appears dispensable for its function on the *toxT* promoter and its role is to enhance the transcriptional activation activity of TcpP, perhaps by recruiting TcpP to a weak promoter-binding site.

Influence of HLA-DR on the phenotype of $CD4^+$ T lymphocytes specific for an epitope of the 16-kDa α-crystallin antigen of *Mycobacterium tuberculosis*

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T helper phenotype may be influenced by cytokine milieu, the differential expression of costimulatory molecules, antigen dose, and by differences in affinity at the TCR-peptide-MHC interface. We investigated the latter hypothesis by examining the response of six HLA-DR-restricted $CD4^+$ T cell lines specific for the immunodominant and permissively recognized p91-110 epitope of the 16-kDa α-crystallin protein of *Mycobacterium tuberculosis*. Each line was generated from a sensitized HLA-DR-heterozygous donor and all proliferated when peptide was presented by autologous irradiated peripheral blood mononuclear cells. However, when HLA-DR-matched homozygous Epstein-Barr-virus-transformed B cell lines (L-BCL) were used as peptide-presenting cells there was heterogeneity in the response. The most pronounced proliferative response, and the highest IFN-γ secretion and cytolytic activity was stimulated by L-BCL expressing molecules (DRB1*0101, *1501 and *0401) with high affinity (IC_{50}< 10 μM) for the 16p91-110 peptide. By comparison, IL-4 secretion or a lower proliferative response could occur when peptide was presented by alleles of high, or of intermediate (10 μM<IC_{50}<100 μM), affinity. These data support the hypothesis that the host MHC can influence $CD4^+$ phenotype and have implications for subunit vaccination against tuberculosis.

COORDINATE EXPRESSION OF STATIONARY PHASE AND VIRULENCE TRAITS: THE ROLE OF RPOS IN LEGIONELLA

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The gram negative bacterium *Legionella pneumophila,* which causes the severe pneumonia Legionnaire's disease, is an intracellular pathogen of amoebae and alveolar macrophages. Previously we found that *L. pneumophila* switches from a replicative to a virulent form in response to amino acid starvation or accumulation of ppGpp, the second messenger which coordinates the *E. coli* Stringent Response. Thus, after exhausting the local nutrient supply, *L. pneumophila* stops replicating and expresses traits which promote transmission, including cytotoxicity, motility and evasion of phagosome-lysosome fusion. In *E. coli*, ppGpp positively regulates expression of the alternative sigma factor RpoS, which induces many genes important for survival in unfavorable conditions. Therefore, we investigated whether RpoS coordinates *L. pneumophila* virulence expression in response to starvation.

The *L. pneumophila rpoS* locus was cloned by complementation of an *E. coli rpoS* mutant by screening for genomic clones which restored expression of a *bolA-lacZ* reporter. Nucleotide sequence analysis revealed a locus with significant homology to *rpoS* of other species (e.g., 71% amino acid identity with *P. aeruginosa* RpoS). Next, we tested whether the *L. pneumophila rpoS* clone complemented a candidate regulatory mutant. The avirulent mutant Lp120 is defective for expression of every virulence trait measured, yet is competent to synthesize ppGpp, suggesting that it lacks a downstream global regulator. Indeed, virulence expression by mutant Lp120 was partially complemented by the genomic fragment containing *rpoS*. Specifically, growth-phase regulated flagellin expression and Na^+ sensitivity were restored, as measured by *flaA-gfp* fluorescence and colony formation on medium containing 100 mM NaCl, respectively. A second indication that Lp120 is an *rpoS* mutant was obtained by PCR analysis: the Lp120 *rpoS* locus contains a deletion of ~100 bp.

RpoS appears to coordinate expression of at least a subset of *L. pneumophila* virulence genes important for infection of macrophages. Further molecular genetic analysis of the interaction between ppGpp, RpoS, and virulence trait expression will establish how *L. pneumophila* responds to environmental signals by regulating effector functions required to colonize aquatic habitats and the human lung.

FUNCTIONAL ATTRIBUTES OF MICROBIAL HEMOGLOBIN-HEME UPTAKE RECEPTORS

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Bacteria face an extremely iron-restricting environment in vivo and must look to host iron reservoirs for survival. Heme is the most abundant reservoir of iron in the human body and numerous pathogens have developed receptors to exploit heme as an iron source. We study two different receptors, HemR from *Yersinia enterocolitica* and HmbR from*Neisseria meningitidis*, which enable bacteria to utilize heme from heme-binding proteins as iron and porphyrin sources. Our work has shown that these heme utilization receptors, while similar to each other in general structure, differ markedly from each other in several fundamental aspects. HemR imports heme from a broad spectrum of substrates. HmbR, on the other hand is adapted specifically to extract heme from Hemoglobin (Hb). We have used genetic and biochemical approaches to isolate discrete receptor functionalities and to identify functionally important residues and domains within the receptor molecules. Site-directed mutagenesis of the four histidine residues in HemR identified two histidines which are essential for the transport function of that receptor. These histidine mutant HemR receptors still bind heme and hemoglobin but fail to transport heme into the bacteria, demonstrating that heme transport and substrate binding are isolated functions for HemR. Deletion analysis of HmbR yeilded two classes of mutant receptor which are unable to utilize Hb as a source of iron and porphyrin. Deletion of a central region results in a mutant receptor which neither binds Hb nor utilizes Hb for growth. Affinity chromatography using Hb-agarose to capture HmbR fragments expressed as fusions to the maltose-binding-protein had previously identified this region to be involved in Hb binding. In contast, an HmbR mutant with a lesion in the amino-terminal pore-plug domain of the receptor resulted in loss of Hb-utilization proficiency but retained binding of Hb. Unlike HemR, no mutants were found for HmbR which were unable to utilize free heme. Also unique to HmbR, high concentration of free heme in vitro overcomes TonB dependence for heme uptake. HemR and HmbR, members of the same family of TonB dependent outer membrane iron uptake receptors, appear to be diverging evolutionary solutions to the task of heme assimilation. *Y. entericolitica*, a pathogen which colonizes a wide variety of environments, prefers a receptor which is open to acquire heme from whatever source it might encounter. *N. meningitidis*, on the other hand, is an obligate human pathogen and has thus evolved a receptor which is strongly adapted to Hb, the most common source of heme in vivo.

THR ROLE OF *HAA1* GENE PRODUCT IN *CANDIDA ALBICANS* DIMORPHISM

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Candida albicans is a dimorphic fungal pathogen undergoing a transition from yeast to mycelial form during a host infection. Two signaling pathways, a MAP kinase pathway and a Ras/cAMP dependent pathway, are reported to be involved in germ tube formation. In *Saccharomyces cerevisiae*, the Ras/cAMP pathway is a nutrient regulated signaling pathway involved in cell division, sporulation and cell growth. In *C. albicans*, the Ras/cAMP pathway is poorly understood. The cAMP dependent pathway, however, is believed to be a key regulatory pathway for the bud-hypha transition and for the pathogenesis of *C. albicans*.

An adenylate cyclase-associated protein (Cap) is one of the main components in the cAMP dependent signaling pathway. Cap is multifunctional protein, acting on the cAMP dependent pathway and on actin cytoskeletal organization. Previously, we cloned and identified a homologue of CAP in *C. albicans*, named *HAA1* for Homologue of Adenylate cyclase Associated protein 1. Haa1p was 40% identical to *S. cerevisiae* Cap. Common features found in other Cap homologues were also observed in Haa1p. Two proline-rich regions in the middle domain and a highly conserved COOH-terminal region were found in Haa1p. Furthermore, Haa1p had a unique NH_2-terminal region representative of the diversity found in Cap homologues from other organisms.

To study the function of Haa1 in *C. albicans*, homozygous *haa1/haa1* mutant strains were constructed using the *URA3* blaster method. Site specific replacement of the *HAA1* gene at the chromosomal locus was verified by Southern hybridization and colony PCR. The *C. albicans haa1* null mutants did not show phenotypes that are typically associated with CAP mutants of other yeasts. The *haa1* mutant strain had the ability to grow on both rich medium (YPD) and minimal medium (YNB, 50mM glucose) and did not show sensitivity to temperature (37°C) and nitrogen starvation. Furthermore, the *haa1* mutant did not show significant morphological abnormalities in the yeast phase. However, preliminary results suggest that *C. albicans* employs the Haa1p to respond to conditions that cause the bud-hypha transition. The *haa1/haa1* mutant strain was defective in germ tube formation under several germ tube-inducing conditions such as M199 at 37°C, Lee's media (pH6.8) at 37°C and YPD containing 20% serum at 37°C. Thus the effect of Haa1p on the bud-hypha transition appears not to be media-conditional, suggesting that Haa1p is important for dimorphism in vivo. Understanding the role of Haa1p in *C. albicans* dimorphism will provide important insight into the pathogenesis of candidiasis.

STUDIES OF THE *IN VIVO* VIRULENCE DETERMINANTS OF UROPATHOGENIC *E. COLI* BY SIGNATURE-TAGGED MUTAGENESIS.

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Uropathogenic *E. coli* (UPEC) is the major cause of urinary infections ranging from bacteriuria to cystitis and pyelonephritis. A few virulence factors have been attributed to this organism, although their roles in pathogenesis remain controversial. In this study, we have adopted large scale isolation of virulence genes by signature-tagged mutagenesis (STM) to identify genes that are essential for UPEC survival within the host urinary tract. We have used STM in the pyelonephritis strain CFT073. This strain harbors two pathogenicity islands on the chromosome and is highly virulent in CBA mouse model of ascending urinary tract infection. A library of 1800 transposon mutants of strain CFT073 was constructed using mini-Tn5Km2 carrying 92 unique tags. Pools of 45-47 mutants were inoculated at 10^8 transurethrally in female CBA mice. After 48h, bacteria were recovered from kidneys and bladders of the animals. PCR-amplified tags from the inoculum and from the recovered bacteria were compared by hybridization with the dot blots of the corresponding tagged plasmids. Screening of 540 mutants resulted in identification of 14 mutants with reduced hybridization signals of the tags. These mutants were individually tested for competition with the wild-type strain in CBA mice. Eleven of the mutants were recovered in numbers 10-10^5 fold less than the wild-type, suggesting attenuation of their growth in animal organs. The interrupted genes were isolated by arbitrary PCR, sequenced and searched for homology by BLAST. Among these mutants, mutations were found in: (i) type 1 fimbrial genes, validating our STM approach; (ii) genes involved in phosphate transport; (iii) genes coding for capsule biosynthesis; (iv) genes involved in O-antigen biosynthesis and (v) genes with unknown function. Two of the genes are absent in the genome of the K-12 strain. The results suggest that STM is a reliable tool for discovery of the virulence genes and potentially novel genes of UPEC.

PHOSPHOLIPASE D1 IN CANDIDA ALBICANS MORPHOGENESIS

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Phospholipase D is a ubiquitous enzyme that plays important roles in phospholipid metabolism, lipid signal transduction pathways and secretion. *Candida albicans* possesses at least one form of phospholipase D, which has been designated CaPLD1. Earlier studies in this laboratory have revealed that CaPLD1 is a calcium-independent, PIP2-stimulated, oleate-sensitive form of PLD. These studies also demonstrated that CaPLD1 was important to the process of dimorphic transition. In the present study, pharmacological agents were used to further support a role for CaPLD1 in dimorphic transition. The drug propranolol was used to inhibit phosphatidate phosphohydrolase, the enzyme which can convert phosphatidate (PA) generated by PLD to diacyglycerol (DAG). Propranolol was able to inhibit the appearance of germ tubes by decreasing the amount of DAG derived from CaPLD1 activity. The DAG was required for a process that was independent of protein kinase C activation, the classic role for DAG. PKC1 activity was inhibited with staurosporine and found to have no effect on dimorphic transition. A possible role for DAG in dimorphic transition will be presented. The anti-fungal undecylenic acid (UDA) was found to inhibit the appearance of germ tubes at sublethal concentrations. A possible mechanism by which UDA inhibits transition will also be presented. In a related series of experiments, it was discovered that resilient denture liners can dramatically effect the morphology of *C. albicans*, possibly affecting its pathogenicity.

LINKAGE OF VIRULENCE AND THE OXIDATIVE STRESS RESPONSE IN *LEGIONELLA PNEUMOPHILA*

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Introduction and Rationale. From the observation (Byrne & Swanson, 1998) that starvation is a prerequisite to expression of virulence traits in *L. pneumophila* we hypothesized that genes required for virulence will be among those required in stationary phase. We initiated studies of catalase/peroxidase because resistance to H_2O_2 is a common trait of bacterial stationary phase and the *rpoS* regulator of the enteric starvation response was identified by its regulation of H_2O_2 resistance. **Results.** *L. pneumophila* contains 2 bifunctional catalase-peroxidases, KatA and KatB. Null mutants of *katA* or *katB* and a double null behave similarly in infection of human macrophage-like cell lines. Each is delayed ≈2 days in the increase of *L. pneumophila* titer, explicable by impairment of either invasion, intracellular replication and/or lysis. The 2 catalase-peroxidases are similar in catalatic activity and peroxidatic activities, however KatB resides in the cytosol while KatA is periplasmic. This compartmentalization suggests that KatA and KatB serve different roles in H_2O_2 decomposition and/or peroxidatic oxidation. KatA is the predominant activity in stationary phase and is required for stationary phase survival. Nulls in *katA* are decreased in survival by 5 orders of magnitude compared to wild type or *katB* nulls after 5 days. **Significance.** Requirement for an antioxidant enzyme in stationary phase is enigmatic because aerobic respiration—the source of 90% of H_2O_2 in rapidly growing cells—is greatly decreased during starvation. We previously observed that *L. pneumophila* copper-zinc superoxide dismutase, another periplasmic antioxidant enzyme, is also required for stationary phase survival. In sum, our studies suggest that periplasmic antioxidant enzymes may play a role in infection of macrophage lines and present efficacious phenotypes for identification of other genes linking the starvation and virulence responses of *L. pneumophila*. (supported by NSF Grants MCB 9513706 & 9809902)

BACTERIAL INFECTION OF DROSOPHILA: A MODEL FOR DISSECTING HOST-PATHOGEN INTERACTIONS.

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In response to microbial infection, Drosophila expresses a battery of antimicrobial peptides genes that are controlled by signalling pathways that share similarities with the pathways that regulate innate immune responses in mammals (e.g.: Toll, rel/NF-kB proteins). A feature of the Drosophila immune response is the ability to differentiate between various classes of microorganisms and to activate specific defense signalling pathways : natural fungal infection activates the Toll signalling pathway that regulates the expression of genes with antifungal activity. To further study the mechanisms that regulate defense response against bacterial infection, we have identified some strains of the bacterial genus, Erwinia, that infect Drosophila and induce a systemic immune response. Erwinia species are phytopathogenic bacteria of the Enterobacteriaceae family that often utilize insects vectors for transmission. Our data indicate that infectious Erwinia species persists for several days inside in the Drosophila host gut and probably cross into the hemolymph. We have found that two Drosophila mutants that lack circulating hemocytes fail to express antibacterial peptide genes after natural infection by Erwinia, thereby, implicating blood cells in the regulation of Drosophila antimicrobial responses. To identify the genes that mediate Drosophila-Erwinia interactions, we are screening for mutations in both the bacteria and its host that block infection. Preliminary results on these mutagenesis experiments will be presented.

ISOLATION OF *Vibrio cholerae* O395 MUTANTS EXHIBITING INCREASED *toxT* EXPRESSION IN NON-INDUCING ENVIRONMENTAL CONDITIONS

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ToxT, a member of the AraC family of transcriptional regulators, controls the expression of several virulence factors in *Vibrio cholerae*. In the classical biotype of *V. cholerae*, expression of *toxT* is regulated by the same environmental conditions that control expression of virulence genes, like cholera toxin and the toxin co-regulated pilus. Several genes that directly or indirectly regulate *toxT* expression have been identified and include *toxR/S*, *tcpP/H* and *cya/crp*. The *toxR/S* and *tcpP/H* gene products have been shown to be activators of *toxT* expression, while the *cya/crp* gene products are the only negative regulators of *toxT* identified so far. To identify additional genes involved in regulation of *toxT* expression, we constructed a strain containing a *toxT*-promoter transcriptional fusion with the reporter gene *gusA* (encoding β-glucuronidase) at the *V. cholerae lacZ* locus and screened Tn*phoA* mutants for colonies showing elevated *toxT-gusA* expression in non-permissive environmental conditions. We were able to identify several mutants showing higher *toxT-gusA* expression relative to the isogenic wild type strain. The mutants could be classified into three classes based on their phenotypic characterization. One class of mutants showed elevated *toxT-gusA* expression at the non-permissive temperature of 37 degree C; a second class of mutants showed higher expression of *toxT-gusA* at pH 8.4, which is normally non-permissive for *toxT* expression; and the third class of mutants exhibited elevated *toxT-gusA* expression at both the non-permissive pH and temperature. Identification of the mutations causing these phenotypes and further characterization of the mutants, which are currently in progress, should enable us to gain insights into the mechanisms involved in the environmental regulation of virulence genes in the classical *V. cholerae* strains.

BFPE, A CYTOPLASMIC MEMBRANE PROTEIN REQUIRED FOR BIOGENESIS OF THE EPEC BUNDLE-FORMING PILUS

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Enteropathogenic *E. coli* (EPEC) produces the bundle forming pilus (BFP), a type IV fimbria that has been implicated in EPEC virulence, autoaggregation, and localized adherence to epithelial cells. A cluster of fourteen *bfp* genes is sufficient to direct BFP synthesis when introduced into non-piliated laboratory strains of *E. coli*. A nonpolar mutation in *bfpE*, the seventh gene of this cluster, abolishes BFP formation and BFP-dependent phenotypes. The *bfpE* gene product, BfpE, is a member of the GspF family of polytopic membrane proteins that function in macromolecular transport pathways of diverse bacteria. BfpE contains four prominent hydrophobic segments. We have sought to experimentally determine the topology of BfpE in the cytoplasmic membrane by constructing plasmids carrying fusions of alkaline phosphatase (*phoA*) or beta-galactosidase (*lacZ*) reporter genes to various *bfpE* derivatives. The *phoA* and *lacZ* genes encode enzymes that are active only when localized to the periplasm or cytoplasm, respectively. The expression of each of the BfpE-PhoA and BfpE-LacZ proteins was analyzed by immunoblotting, and their enzyme activities were quantified to suggest the cellular location of the fused enzyme moiety. These analyses support a topology in which BfpE contains three transmembrane domains. A fourth hydrophobic domain is capable of crossing the membrane, yet does not appear to do so in the context of the full-length protein. BfpE contains two small periplasmic domains and two large cytoplasmic domains, which are sites for potential interactions with other Bfp proteins. These findings concerning the topology of BfpE provide a structural basis for further study of the role of this component in the BFP synthesis machinery.

IDENTIFICATION OF A COMPLEX GROUP I INTRON IN A EUBACTERIAL PATHOGENICITY FACTOR

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Within *tcdA-C34,* the enterotoxin gene of the clinical *Clostridium difficile* isolate C34, a DNA insertion of 1975 bp was identified that we designated CdtcdA. Located in the catalytic domain A1-C34, CdtcdA combines features of two genetic elements. Within the first 434nt the secondary structure (P1 to P9) and conserved nucleotides characteristic for group I introns are found. Nucleotides 435 to 1975 contain open reading frames coding for two transposase like proteins (TlpA/B) with homology to transposases reported for IS elements of the IS*605* group. RT-PCR analysis proved splicing of the entire CdtcdA from *tcdA1-C34* primary transcripts, and intramolecular cyclization of excised CdtcdA. In consequence purified TcdA-C34 toxin was of regular size and catalytic activity. CdtcdA is the first group I intron ever found in a eubacterial gene other than tRNA or phage mRNA-encoding genes. Screening three toxinogenic and one nontoxinogenic *C. difficile* strains for CdtcdA homologous sequences, we found 4-11 copies in each genome. Its composite structure and localisation in eubacterial genes (other than tRNA and phage genes) discriminates CdtcdA from other known group I introns. We hypothesise that CdtcdA constitutes a novel group of chimeric mobile elements adapted to spread in eubacterial genomes.

SECRETED PROTEINS AND SECRETION MECHANISMS OF *M. TUBERCULOSIS*

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Since secreted proteins and cell envelope-associated proteins are likely to participate in the virulence of *M. tuberculosis* and in the generation of protective immunity to *M. tuberculosis*, we are interested in identifying and characterizing both secreted proteins and the export pathways responsible for their localization.

In order to identify secreted proteins of *M. tuberculosis*, we have developed a Tn*552phoA in vitro* transposition system. Specifically, we constructed a Tn*552phoA* transposon, which can efficiently and randomly transpose into mycobacterial DNA *in vitro*. Using this transposon, *in vitro* transposition reactions were carried out on a genomic cosmid library of *M. tuberculosis*. The resulting cosmids were introduced into *M. smegmatis* and screened for active PhoA fusions. Since the *phoA* gene on the transposon lacks a signal for secretion, active PhoA fusions indicate in-frame fusions to exported proteins. To date, we have identified approximately 50 different active PhoA fusions to *M. tuberculosis* proteins; many of these exported proteins have not previously been described. We are continuing to screen for additional exported proteins of *M. tuberculosis*. In addition, we are using the transposon insertions as substrates for allele exchange in *M. tuberculosis* to create mutants lacking individual secreted proteins. These mutants will enable us to evaluate the role these proteins play in virulence and protective immunity.

Our analysis of the secretion pathways operating in *M. tuberculosis* currently focuses on the SecA protein. A unique feature of the mycobacterial secretion pathway is the presence of two *secA* homologues (*secA1* and *secA2*). Both *secA1* and *secA2* are highly homologous to the *secA* genes of other organisms; however, the proteins encoded by these two genes are only 35% identical to each other. Using *M. smegmatis*, we have begun to characterize the two SecAs. We have determined that *secA1* is essential (like SecA in *E. coli*), while *secA2* is not. This result and others indicates that the two SecAs are not functionally equivalent. Yet, the phenotype of a *secA2* in-frame deletion mutant suggests that SecA2 participates in secretion. We are further studying this mutant to characterize the role that this "extra" SecA plays in mycobacteria. We are also constructing a deletion of the *secA2* gene in *M. tuberculosis*. Such a mutant will enable us to analyze the role that a secretion pathway plays in *M. tuberculosis*.

CONTRIBUTION OF TOXINS AND ROLE OF THE ADJUVANT EFFECT OF THE PROTECTIVE ANTIGEN (PA) IN ANTHRAX PATHOGENESIS.

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Bacillus anthracis, a spore forming gram positive bacterium, is the causative agent of anthrax. The virulence of the bacterium is essentially due to the synthesis of two toxins and one capsule. The lethal toxin (Letx), composed of the lethal factor (LF) and the protective antigen (PA), causes death in animals. The edema toxin composed of the edema factor (EF) and PA induces an edema formation at the inoculated site. *B. anthracis* strains harboring point mutations or small deletions in the toxin gene components were constructed. Spores of the recombinant strains were injected to mice and the lethality, the edema formation and the humoral response directed against PA and LF were analyzed. The *in vivo* properties of the mutated strains indicate a correlation between the toxin mode of action and the infectious process. Moreover, we showed that the antibody titers against LF were significantly higher when the strain produced a PA molecule able to bind LF *in vivo*. This effect of PA was not dependent of the enzymatic activity of the Letx. The adjuvant effect of PA has also been observed against an heterologous antigen: the C fragment of tetanus toxin (ToxC) fused to the N-terminal part of LF which is required for the binding to PA. Indeed, the *B. anthracis* strain producing both PA and the hybrid protein LF-ToxC induced a humoral response against ToxC significantly higher than that observed with the strain producing LF-ToxC but not PA. Moreover, recombinant strains producing ToxC are able to protect mice against a lethal challenge with tetanus toxin.

THE ROLE OF NEUTROPHILS IN EXPERIMENTAL LYME ARTHRITIS

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The innate immune response can mediate both resistance and susceptibility to experimental Lyme arthritis. In vitro, neutrophils have been shown to take up and kill *Borrelia burgdorferi* and to secrete a number of pro-inflammatory compounds. Although neutrophils can be recovered from synovial fluid of patients with active Lyme arthritis, and constitute a large part of the inflammatory infiltrate found in the joints of experimental animals, their role in mediating the pathology of Lyme disease remains unknown. To determine their in vivo role in experimental Lyme arthritis we depleted both arthritis-resistant DBA/2 and arthritis-susceptible C3H/HeJ mice of neutrophils using daily injections of the monoclonal antibody RB6-8C5. Peripheral blood was monitored for neutrophil depletion and arthritis development was followed for 21 days.

All neutrophil-depleted mice had exacerbated inflammation of their tibiotarsal joints during the first week of infection regardless of mouse strain. During the second and third weeks of infection arthritis development was similar between the neutrophil-depleted and control C3H animals. Arthritis development in the neutrophil-depleted DBA mice approached that of the susceptible C3H mice. The control DBA mice did not develop arthritis at any time during the experimental period. Spirochete numbers in tissues were similar between control and neutrophil-depleted animals.

These results indicate that neutrophils provide protection against early inflammatory responses in both arthritis-resistant and -susceptible mouse strains. During the second week of infection this protection is overcome in arthritis-susceptible, but not arthritis-resistant strains. This protection is not mediated through neutrophil bactericidal activity since both control and neutrophil-depleted animals harbored relatively equal levels of spirochetes in their tissues. Finally, neutrophils do not appear to be contributing to pathology since arthritis development was similar in control and neutrophil-depleted C3H mice.

PURIFICATION AND CRYSTALLIZATION OF THE CATALYTIC DOMAIN OF CYTOTOXIC NECROTIZING FACTOR 1 (CNF1), A BACTERIAL PATHOGEN PROTEIN THAT MODIFIES THE HOST CYTOSKELETON

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Cytotoxic necrotizing factor 1 (CNF1), a 1014 amino acid toxin encoded by a pathogenicity island of uropathogenic strains of *E. coli*, is a member of a recently recognized superfamily of toxins that affect the actin cytoskeleton of host cells. Among these toxins CNF1 has a novel catalytic activity. The toxin acts by deamidating a specific residue (Gln 63) of the small G-protein RhoA and thereby abrogating the GTPase activity of RhoA. This constitutive activation of RhoA caused by CNF1 results in changes in the host cell architecture, including formation of stress fibers and membrane ruffling, and is also involved in bacterial invasion of nonphagocytic host cells.

CNF1 has two domains essential to its function. The N-terminus (residues 1-299) is responsible for binding to a host cell receptor while the C-terminus (720-1014) is necessary for catalytic function of CNF1. The C-terminus of CNF1 has homology to the dermonecrotic toxin (DNT) of *Bordetella* spp. and the human microtubule associated protein 4 (MAP4). We are focusing on understanding the molecular mechanism for the recognition and catalytic deamidation of RhoA by the C-terminal domain of CNF1 by x-ray crystallographic techniques. We present here the overexpression, purification, characterization, and crystallization of this catalytic domain. The structure of this domain will be important to understanding the mechanisms used by pathogens to alter the behavior of host cells.

MOLECULAR AND EVOLUTIONARY ANALYSIS OF THE CP32/18 FAMILY OF SUPERCOILED PLASMIDS IN *BORRELIA BURGDORFERI* 297

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Borrelia burgdorferi, the causative agent of Lyme disease, is maintained in an enzootic cycle involving *Ixodes scapularis* and a mammalian host, typically rodents. There is now a substantial body of evidence that the transition from vector to host is accompanied by marked changes in antigenic composition and that this is accomplished predominantly by differentially expressed plasmid-encoded genes. In this regard, the 32-/18-kb family of *B. burgdorferi* supercoiled plasmids (cp32/18) has garnered much attention. *B. burgdorferi* isolates contain up to nine distinct cp32/18 plasmids, each consisting of large, highly conserved stretches and two hypervariable regions encoding the differentially OspE/OspF/Elp and 2.9 lipoproteins, respectively, as well as other ORFs. Here we used a combination of DNA sequencing along with long-distance and routine PCR as the basis for a comprehensive analysis of the cp32/18 family. A region encoding a MinD/Spo0J homolog, multiple dnaA boxes, and a putative replication protein-binding site is likely to be the origin for a novel theta-type mechanism of replication. The cp18 plasmids lack a 13 kb piece of DNA situated between the two lipoprotein-encoding loci; none of the presumably non-essential ORFs on these fragments matched other sequences in the databases. Pairwise analysis with random shuffling revealed that the 2.9 lipoproteins fall into two distinct classes which we theorize arose by gene fusion events similar to those previously proposed to have generated the OspE/OspF/Elp proteins. Comparison of the strain 297 cp32/18 sequences with those recently obtained for the Medimmune B31 clone revealed three notable differences: (i) some OspF/Elp/Erp loci in one strain contain ORFs not present in the other, (ii) the B31 plasmids contain only one class of 2.9 lipoproteins, and (iii) the strain 297 lipoproteins show much more sequence diversity than their B31 counterparts. This work underscores the prominent role of recombination in the evolution of these plasmids and the generation of antigenic diversity among the differentially expressed lipoproteins they encode.

IDENTIFICATION OF VIRULENCE GENES OF MYCOBACTERIUM TUBERCULOSIS BY SIGNATURE-TAGGED TRANSPOSON MUTAGENESIS

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Tuberculosis remains the greatest cause of death worldwide due to a single pathogen. In order to identify the genes required for the pathogenicity of *M. tuberculosis*, a functional genomic approach was developed. A library of signature-tagged transposon mutants of this bacterium was constructed and screened for those affected in their multiplication within the lung of mice. From 1927 mutants tested, sixteen were attenuated for their virulence. The insertions harboured by the selected mutants were mapped on the *M. tuberculosis* genome and most of the mutated loci appeared to be involved in lipid metabolism or transport across the membrane. Four independant mutations identified a cluster of virulence gene located on a 50 kb chromosomal region. These genes might be involved in the production a group of molecules restricted to pathogenic mycobacterial species. The interaction of five mutant strains with mouse bone-marrow macrophages was investigated. These five mutants were still able to multiply in this cell type. However, in three cases, there was a growth defect in comparison to the wild type strain. The other two strains exhibited no clear difference with the virulent strain MT103 in this model. This study which is the first global research of virulence factor of *M. tuberculosis* opens the way to a better understanding of the molecules that are key player in the interaction of this pathogen with its host.

A NOVEL ACONITASE GENE IN THE PROPIONATE OPERON OF PATHOGENIC BACTERIA *PSEUDOMONAS AERUGINOSA, NEISSERIA GONORRHOEAE, NEISSERIA MENINGITIDIS AND BORDETELLA PERTUSSIS*

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Short-chain fatty acids such as acetate, propionate and butyrate induce stress responses and enhance virulence in bacteria. Pathogenic bacteria encounter various concentrations of these naturally occurring short-chain fatty acids in their environment. The formation and degradation of most short-chain fatty acids involve β-oxidation with the exception of propionate, which is mainly produced as a by product of odd-chain fatty acid degradation and catabolized possibly by several different pathways. Here we report that *Pseudomonas aeruginosa* has a novel aconitase gene (*acnC*) which is localized with a cluster of genes involved in the catabolism of propionic acid. The order of the genes in the *P. aeruginosa* propionate operon (*prp* operon) is similar to *E. coli* and *Salmonella prp* operons, with the exception of *acnC* and *yraM* genes, which are not present in *E. coli* and *Salmonella*. The *P. aeruginosa prp* operon contains the genes encoding isocitrate lyase, methylcitrate synthase, aconitase (*acnC*) and homologs for the hypothetical genes *yraM* and *prpD*. The novel aconitase *acnC* is possibly involved in the interconversion of methylcitrate and methylisocitrate. Sequence analysis of *acnC* shows that amino acid residues implicated in iron-sulfur cluster binding, substrate recognition and those potentially involved in catalytic as well as RNA-binding activities are highly conserved. However, phylogenetic comparison of *acnC* to known aconitases suggests that *acnC* might have evolved divergently to fulfill a specific function. Analysis of the available microbial genome sequences shows the existence of *acnC* homologs in identical contexts of propionate catabolizing operons in at least three other pathogenic bacteria. Insertional disruption of *P. aeruginosa* PAO1 *acnC* gene completely abolished growth in media containing 0.1% propionic acid. The mutant exhibits ~25% less aconitase activity than the wild type strain PAO1. In addition, compared to wild type, the *acnC* mutant shows notable attenuation in hemolytic, proteolytic and elastase activities. These observations indicate that propionic acid metabolism in *P. aeruginosa* probably relies mainly on the methylcitrate pathway involving *acnC*. This aconitase may control the expression of certain virulence genes. These results also suggest that odd-chain fatty acids may contribute to the induction of virulence factor synthesis.

A *PSEUDOMONAS AERUGINOSA* VIRULENCE GENE ENCODES A LYSR-LIKE TRANSCRIPTION REGULATOR OF *PHNA/B* OPERON

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The UCBPP-PA14 strain of *Pseudomonas aeruginosa* has been shown to infect both plants and animals. Ten Tn*phoA*-mutagenized mutant strains have been isolated which exhibit significantly reduced pathogenicity in both plant and animal hosts. One mutant, *phoA 34B12* has been characterized in detail in this study. The mutant grows about 10-fold less than wild type strain in *Arabidopsis thaliana* ecotype LI-0 and its mortality rate in a mouse full-thickness skin burn model is reduced by 50%. In addition, it was shown that the disruption of the 34B12 gene affects a number of virulence-related factors, including the production of pyocyanin, elastolytic and hemolytic activities. The pleiotropic effect of the mutation indicates that the gene is likely to be a regulatory factor for the pathogenicity of *P. aeruginosa*. Sequence analysis revealed that there are two ORFs (designated as ORF1 and ORF2) in the region containing the mutation, and the two ORFs are oriented in the opposite direction, nearly fully overlapping with each other. A single non-sense point mutation was introduced into each ORF and phenotypic analysis of the point mutants identify ORF2 as the regulatory factor. More detailed sequence analysis of ORF2 indicate that it contains at its N-terminus the signature helix-turn-helix (HTH) motif which belongs to the LysR family of transcription regulators. RNA blot analysis showed that the transcription of *phnA/B* operon, the genes encoding anthranilate synthase involved in pyocyanin biosynthesis, is significantly reduced when ORF2 is mutated. Gel shift assay demonstrated that ORF2 binds specifically to a 51-bp sequence in the promoter region of *phnA/B* operon. Taken together, these data indicate that ORF2 is a LysR-like transcription regulator of *phnA/B* operon. Interestingly, a signal peptide sequence was found at the N-terminus of ORF2 and its putative cleavage site overlaps with the HTH motif. Experiments are being carried out to uncover the regulatory role of the signal peptide.

ABOLITION OF THE RESPIRATORY BURST RESTORES THE ABILITY OF *SLYA* MUTANT *SALMONELLA TYPHIMURIUM* TO GROW IN MACROPHAGES AND CAUSE LETHAL INFECTION IN MICE.

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Recent studies have demonstrated that *Salmonella* must be able to counter toxic oxidative products generated by the respiratory burst of host macrophages, most notably superoxide and hydrogen peroxide. Genes have been identified that are required for Salmonella growth in macrophages, but of these only *recA/recBC*, *sodC*, and *slyA* thus far have been shown to be specifically involved in resistance to oxidative stress. Our laboratory has shown that the *slyA* gene, directing the expression of the SlyA regulon, is absolutely required for virulence in mice and growth in macrophages. We have found that the SlyA regulon is required for resistance to exogenously administered hydrogen peroxide and intracellular superoxide generated by paraquat. To demonstrate the in vivo sensitivity of *slyA* mutant S.typhimurium to phagocyte-related oxidative stress, we infected gp91phox ko (knock-out) mice which are unable to generate a respiratory burst due to a mutation in the 91kD membrane glycoprotein of cytochrome b558. Mice that were infected with slyA mutant S.typhimurium succumbed to lethal infection, while the isogenic parent C57BL/6 mice were resistant to the same inoculum. Peritoneal macrophages from the gp91phox ko mice were permissive for *slyA* mutant growth, but macrophages from congenic C57BL/6 mice were not. These data provide in vivo support for the hypothesis that the SlyA regulon plays an essential pathogenic role by defending Salmonella against toxic oxidative compounds produced by host phagocytic cells.

IDENTIFICATION OF DNA SEQUENCE MOTIFS IN VIRULENT GENES OF GROUP A STREPTOCOCCI THAT PREFERENTIALLY STIMULATE HUMAN LYMPHOCYTES

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Background. Immunostimulatory oligodeoxynucleotides (ODNs) containing CpG motif (CpG dinucleotides within specific base context) can induce proliferation of murine and human B cells and elicit inflammatory cytokine production. To investigate a potential role of bacterial DNA in the immunopathogenesis of group A streptococci infections, we investigated for the presence of stimulatory DNA motifs in streptococcal genes.

Methods. Streptococcal genome database and several streptococcal genes sequences were searched for the presence of the optimal CpG motifs that have been reported to stimulate murine and/or human lymphocytes. ODNs copying streptococcal DNA sequences of interest were then tested in a proliferation assay for ability to stimulate human or murine lymphocytes. The cell populations responding to ODN stimulation were identified by monitoring the expression of the activation marker CD25 and the surface antigens of T and B cells (CD3 and CD20 respectively) by 2-color flow cytometry analysis.

Results. We report that the *emm*1 gene encoding the streptococcal virulence factor M1 protein contains TCG elements that preferentially stimulate human peripheral blood mononuclear cells. ODNs copying the *emm*1 gene sequence which harbors this motif induced the proliferation of human B cells and upregulated the expression of CD25 on their surface. Removal of T cells slightly reduced the proliferative response to the ODNs indicating a direct effect of these motifs on B lymphocytes. Inter-individual variations in responsivness to DNA motifs present in the streptococcal M1 gene were observed, suggesting that host factors may regulate these responses. Interestingly, similar motifs were also found in genes encoding several streptococcal superantigens as well as in genes encoding M-like proteins of group C and G streptococci.

Conclusion. The finding that group A streptococcal DNA contains stimulatory TCG motifs suggests that this is yet another mechanism involved in the immunopathogenesis of invasive group A streptococci infections.

MUCOSAL AND SYSTEMIC IMMUNE RESPONSES TO CHIMERIC FIMBRIAE EXPRESSED BY SALMONELLA VACCINE STRAINS

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Among the various vaccines against infectious agents, recombinant live vaccines expressing pathogen-derived antigens offer a unique set of attractive properties including the simplicity of preparation and administration, and the capacity to induce mucosal and systemic immunity. Live bacterial carrier vaccines have the additional advantage of permitting genetic manipulation for optimal antigen presentation and for targeting preferentially cellular or humoral responses. To determine whether the immune system is more responsive to an intracellular or to an exposed antigen located on the bacterial surface, we compared the systemic and mucosal humoral responses of mice immunized with a *Salmonella* vaccine strain displaying fimbrial antigen on its surface or in its periplasmic compartment, respectively. Orally immunized Balb/c mice showed that 987P fimbriated *Salmonella typhimurium* CS3263 (*aroA asd*; *asd*-derivative of strain SL3261, kind gift of B. Stocker) with pCS151 (*fas*$^+$ *asd*$^+$) elicited significantly higher level of systemic IgGs and mucosal IgAs than *S. typhimurium* CS3263 with pCS152 (*fasD asd*$^+$) expressing periplasmic antigen. Further studies were aimed at determining whether the 987P fimbriae expressed *in vivo* by *S. typhimurium* χ4550 (*cya crp asd*; kind gift of R. Curtiss) could be used as carriers of foreign epitopes (Rani, Bayer and Schifferli, 1999, Clin. Diagn. Lab. Immunol. 6:30-40). For this, the vaccine strain was genetically engineered to express chimeric fimbriae carrying the transmissible gastroenteritis virus (TGEV) C (379-388) and A (521-531) epitopes of the spike protein inserted into a permissive site of FasA, the 987P major fimbrial subunit. Balb/c mice administered orally *S. typhimurium* χ4550 expressing the chimeric fimbriae from the *tet* promoter in stably-maintained plasmid pCS154 (*fas*$^+$)produced systemic antibodies against both fimbria and the TGEV C epitope, but not the TGEV A epitope. In order to improve the immunogenicity of the chimeric fimbriae, the *in vivo* inducible *nirB* promoter was added to the tetracycline promoter to create pCS155. In comparison to the previously used vaccine, Balb/c mice immunized orally with *S. typhimurium* χ4550 pCS155 demonstrated higher levels of serum IgGs and secretory IgAs against 987P fimbria and systemic IgGs against the TGEV C epitope. Moreover, this new vaccine elicited also TGEV C-specific secretory IgAs and TGEV A-specific serum IgGs. Hence, *in vivo* expression can be optimized to improve foreign epitope presentation by a fimbrial carrier system expressed on a *Salmonella* vaccine strain.

A NOVEL ANTIDOTE FOR BOTULISM: A COMPOUND EXTRACTED FROM THE SEED of *Melia toosendan.*

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Botulinum toxin is the most potent toxin in the world. There is no known cure for botulism and the major therapy is antiserum infusion to neutralize toxin or uses supporting therapy to maintain life. *Melia toosendan* is known as both effects of relieving pain in trauma and being a vermifuge in traditional Chinese medicine. In this study, we found a compound, coded with TSN and extracted from the seed of *Melia toosendan,* showed the anti-botulism effect. The results of structure identification indicated that the physical characteristic of TSN was following : (1) molecular weight is 356; (2) chemical formula is $C_{20}H_{20}O_6$; (3) melting point is 280 C°. *In vitro* studies, the electric stimulating contractile force of rat phrenic nerve diaphragm was depressed by botulinum toxin A (1 ng/ml) and the contraction will be completely paralyzed after 4 hours toxin treatment. This inhibition effect of botulism could be antagonized with dose-dependent when TSN (0.1-10 μg/ml) was added to tissue bath. *In vivo* studies, TSN could increase the mouse LD50 of botulinum toxin A intoxication. If mouse was injected with lethal dose of botulinum toxin A (8 ng/kg, ip) and TSN-B (10 mg/kg, ip) was simultaneously treated, the survival rate of intoxication mice would be up to 80%. Even though the mice were intoxicated for 2 hours, TSN also showed the same therapeutic effect. Both *in vitro* and *in vivo* results suggested that TSN-B might be a new antidote for botulism.

HELICOBACTER PYLORI INFECTION IN INTERLEUKIN-4 DEFICIENT AND TRANSGENIC MICE.

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Background: Interleukin (IL)-4 is a potent anti-inflammatory and Th2 type immuno-regulatory cytokine. *Helicobacter pylori* infection in humans induces a polarised Th1 immune response characterised by increased production of interferon-gamma and absence of IL-4. This study was designed to determine the role of endogenous IL-4 in host defence against gastric colonisation by *H. pylori* using IL-4-deficient (IL-4-/-) and transgenic (IL-4 Tg) mice. **Methods:** IL-4-/- mice and IL-4 Tg mice were inoculated intragastrically with *H. pylori* Sydney Strain 1. Gastric colonisation by *H. pylori* (biopsy urease test and bacterial colony counts), serum levels of *H. pylori*-specific immunoglobulin (Ig) M, A, G, isotypes of IgG, and the gastric mucosal inflammatory scores were determined at 6 weeks post-inoculation. Results were compared with those obtained from *H. pylori*-infected IL-4+/+ (controls for IL-4-/- mice) and IL-4 WT (controls for IL-4 Tg) mice. **Results:** Colonisation of gastric mucosa by *H. pylori* in IL-4-/- mice was similar to control IL-4+/+ mice. There was no significant difference in titres of *H. pylori*-specific antibodies or gastric inflammatory scores between the two groups of mice. Colonisation of gastric mucosa by *H. pylori* was consistently lower in IL-4 Tg mice ($\log_{10}$ 6.40 $\pm$ 1.09 CFU/g tissue) compared to IL-4WT mice ($\log_{10}$ 7.20 $\pm$ 0.34 CFU/g tissue) although the difference was not significant. Nevertheless, IL-4 Tg mice did have significantly higher titres of *H. pylori*-specific IgA and IgG ($P \leq 0.01$). **Conclusion:** These results show that endogenous IL-4 is not a major contributor to host resistance to *H. pylori* and enhanced IL-4 production has little of any effect on gastric colonisation by this organism, in spite of elevated specific antibody production. Supported by WMRF, HRC and Lottery Grant Committee of NZ.

REDUCED COLONISATION OF GASTRIC MUCOSA BY *HELICOBACTER PYLORI* IN INTERLEUKIN (IL) 10-DEFICIENT MICE WITH COLITIS

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Background: Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine, and plays an important role in the regulation of mucosal immune responses *in vivo*. Mice deficient in IL-10 production (IL-10-/- mice) develop spontaneous chronic intestinal inflammation. The objective of this study was to determine whether IL-10 deficiency and chronic intestinal inflammation in IL-10-/- mice affects the colonisation of gastric mucosa by *Helicobacter pylori*. **Methods:** IL-10-/- mice and age-matched IL-10+/- mice were inoculated intragastrically with a mouse-adapted *H. pylori* isolate (Sydney Strain 1). The numbers of bacterial colonies in the gastric mucosa, the severity of gastric inflammation and the levels of *H. pylori*-specific antibodies were determined at 2 or 6 weeks post-inoculation. **Results:** Colonisation of gastric mucosa by *H. pylori* was reduced up to 100-fold ($P \leq 0.001$) in IL-10-/- mice with colitis ($\log_{10}$ 4.87 $\pm$ 0.26 CFU/g tissue) as compared to IL-10+/- mice ($\log_{10}$ 6.64 $\pm$ 0.22 CFU/g tissue). Furthermore, those IL-10-/- mice showed significantly higher serum *H. pylori*-specific IgM, IgA and IgG antibodies ($P \leq 0.01$), and developed more severe chronic active gastritis ($P \leq 0.05$) than IL-10+/- mice following *H. pylori* infection. The median scores of gastric inflammation were up to 3 fold higher in IL-10-/- mice with colitis than in IL-10+/- mice. Glandular dilatation and abscesses were only seen in the gastric mucosa from infected IL-10-/- mice with colitis. In contrast, bacterial colonisation, gastric inflammation and specific antibody levels in IL-10-/- mice that did not have colitis were similar to those of age-matched IL-10+/- mice. **Conclusion:** The colonisation of gastric mucosa by *H. pylori* was significantly reduced in IL-10-/- mice with colitis as compared to IL-10+/- mice. However, the bacterial colonisation in IL-10-/- mice without colitis was similar to that in IL-10+/- mice. This suggests that IL-10 *per se* does not play a significant role in host defence against *H. pylori*. The chronic intestinal inflammation secondary to IL-10 deficiency is in some way responsible for the reduced colonisation of gastric mucosa by *H. pylori* seen in IL-10-/- mice. Supported by WMRF, HRC and Lottery Grant Committee of NZ.

INVOLVEMENT OF THE TNF-ALPHA SIGNALING PATHWAY IN HOST CELL RESPONSE TO R. RICKETTSII INFECTION

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Previous work in our laboratory has shown that NF-kappa B activation is required to inhibit apoptosis during in vitro infection with the obligate intracellular bacteria, *Rickettsia rickettsii*. Thus *R. rickettsii* transduces a dual signal during infection resulting in both NF-kappa B activation and apoptosis, similar to that seen in response to TNF-alpha. Studies were undertaken to explore the possible role of the TNF signaling pathway in these *R. rickettsii*-induced host cell responses. When murine lung fibroblasts (FB) cultured from TNF receptor 1knockout (TNFR1-/-) animals were infected with *R. rickettsii*, no NF-kappa B activation was observed at 3, 7, 14 or 21 hours post-infection, whereas biphasic activation peaking at 3 and 18-21 hours was seen in several cell lines, including fibroblasts cultured from normal mice. TNFR1-/- FB rapidly underwent apoptosis when infected, consistent with lack of NF-kappa B activation. Neutralizing antibodies against human TNF-alpha were used to explore its possible involvement in the host response. This antibody, when present on HUVEC prior to and during infection, did not ablate NF-kappa B activation as measured at 3 hours, nor did it block apoptosis when added together with proteasome inhibitors to block NF-kappa B activation. In addition, rickettsial infection of EC did not result in increased levels of TNF-alpha mRNA throughout the time course of infection. These results suggest involvement of the TNFR1 receptor, but not TNF-alpha itself, in *R. rickettsii*-induced signal transduction leading to NF-kappa B activation but not apoptosis.

A CYTOTOXIC CELL VACUOLATING ACTIVITY ASSOCIATED WITH HEMOLYSIN PRODUCTION IN *VIBRIO CHOLERAE*

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A Vibrio cholerae cytotoxin was found to cause vacuolation in Vero cells. The vacuolating phenotype is similar to the effect of the Vac A toxin of Helicobacter pylori on mammalian cells. The vacuoles start to be seen 2 hours after inoculation with a culture supernatant and at 5 hours they are present in high numbers in the cell cytoplasm, filling almost all of the space of the cell. They are quantified at 24 hours, by microscopy, determining the dilution with the highest vacuolation, or by neutral red uptake. At higher concentrations than those used for vacuolation, the cells become rounded and detach from the flask. This toxin was originally identified in the pathogenic O1 Amazonia variant of *V. cholerae*, and later shown to be produced in environmental strains and some El Tor strains. This vacuolating cytotoxin (VcVac) is a secreted, thermo sensitive (55°C), proteinase K sensitive and trypsin resistant protein. Bafilomycin A inhibits the vacuolation caused by VacA, but in contrast with that, VcVac vacuoles are still formed normally, and the cytotoxic activity is still present. A comparison of VcVac production in various strains led to the possibility that the hemolysin was responsible for the vacuolating phenotype, as there was a strong correlation in the El Tor strains between vacuolation and the hemolytic phenotype. The *hlyA* gene of the Amazonia variant was sequenced and shown to have six amino acid differences in the carboxy terminal half of the protein, thought to be the region responsible for the hemolytic activity. Insertion mutations in the *hlyA* gene of the Amazonia strain were prepared, with the use of the suicide plasmid pKAS32 . This insertion abolished both the cytotoxin vacuolating activity and hemolysis. Revertants of these insertion mutants regained both phenotypes, confirming that the lack of activity of the insertion mutants was in fact due to the inactivation of the *hlyA* gene. The involvement of the hemolysin of *V. cholerae* with a mammalian cell vacuolating phenotype is a new property of this protein, and points to a previously unknown type of interaction of *V. cholerae* and its host.

UPTAKE OF *ENCEPHALITOZOON CUNICULI* BY NON-PROFESSIONAL PHAGOCYTES

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Background: *Encephalitozoon cuniculi*, an obligately intracellular, spore-forming protozoan parasite belonging to the Microsporidia can cause disseminated disease involving every organ system in severely immunocompromised humans. *E. cuniculi* spores infect cells by eversion of the polar filament through which the infectious spore content is injected through the cell membrane into the cytoplasm. However, we also found some *E. cuniculi* spores to enter human lung fibroblasts (MRC5 cells) without polar tube discharge. We studied the mechanism of spore uptake by these non-professional phagocytes.

Results: Paraformaldehyde-fixed spores were taken up by MRC5 cells at the same rate as live spores, excluding a mechanism of active invasion. Uptake of *E. cuniculi* spores by human monocyte-derived macrophages reached a plateau 2 hours after infection (89% of added spores internalized). In comparison, internalization by MRC5 cells increased linearly over time (4.5% and 26% internalized spores at 2 and 24 h, respectively). Up to a multiplicity of infection of 100 spores/cell, the rate of uptake increased linearly with the amount of added spores. Cytochalasin D blocked uptake of spores by MRC5 cells by 95%, indicating an actin-dependent process. By electron- and epifluorescence microscopy, intracellular spores were found in a membrane-bound compartment strongly positive for lysosome-associated membrane protein-1. The phagolysosomal nature of the vesicle containing *E. cuniculi* spores was confirmed by the colocalization with rhodamine-dextran, a content marker for lysosomes. Some spores everted the polar filament after uptake, reaching the cytoplasm after penetrating the vacuolar membrane.

Conclusion: Compared to macrophages the rate of uptake of *E. cuniculi* spores by non-professional phagocytes was lower and the kinetics were slower. However, the mechanism of uptake of *E. cuniculi* by MRC5 cells is indistinguishable from phagocytosis. Phagocytic uptake followed by polar tube eversion may enhance infectivity of *E. cuniculi*

THE *VIBRIO CHOLERAE* RS1 ELEMENT IS BOTH A PARASITE AND A HELPER

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CTXϕ is a lysogenic, filamentous bacteriophage found in *V. cholerae*. Its genome includes the genes that encode cholera toxin. CTX prophages are freqently flanked by a related genetic element, RS1. RS1 contains the 3 genes (*rstR, rstA, and rstB*) and the two intergenic sequences (ig-1 and ig-2) that constitute the RS2 region of CTXϕ, and in addition contains *rstC*, a gene not found in the CTXϕ genome. The function and transmissibility of RS1 are not known. We have detected a plasmid form of RS1 in strains that harbor RS1 adjacent to a CTX prophage, suggesting that an independent, extrachromosomal form of RS1 can be generated during *V. cholerae* growth. The replicative capacity of RS1 was confirmed with a suicide vector harboring an RS1 derivative, which was maintained as a plasmid in a strain lacking a CTX prophage and *attRS*, the phage attachment site. After introduction into an *attRS*$^+$, CTXϕ- strain, this plasmid integrated into *attRS*, indicating that RS1 includes the sequences sufficient for site-specific integration at *attRS*. RS1 does not include the 'core' region of the CTXϕ genome – a region required for CTXϕ particle morphogenesis – so our failure to detect transduction of RS1 from this strain was not unexpected. However, we found that RS1 could be efficiently packaged by CTXϕ $^+$ strains. The plasmids found in RS1 transductants did not contain core region sequences, indicating that recombination between the RS1 and CTXϕ genomes does not underly CTXϕ -mediated transduction of RS1. In addition to this parasitic activity, RS1 also enhances the transmissibility of the CTX prophage. Strains that harbor only a single CTX prophage produce few or no CTXϕ transducing particles. The presence of an RS1 element either 5' or 3' of a single CTX prophage leads to a marked enhancement in the titer of CTXϕ transducing particles. Together, these results demonstrate that RS1 is a novel symbiotic genetic element that acts both as a parasite of and helper to CTXϕ and thereby plays a critical role in the transmission of the cholera toxin genes. Preliminary analyses indicate that the molecular basis for the rescue of CTX prophage viability by RS1 is rooted in the mechanism and site of initiation of CTX prophage DNA replication.

HLA-A2-RESTRICTED, CD8-DEPENDENT CYTOTOXIC T-CELL RESPONSE TO MYCOBACTERIAL SUPEROXIDE DISMUTASE.

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Superoxide dismutase (SOD) is a major protein found in supernatants of cultured *Mycobacterium tuberculosis* (*M. tb.*), and it is thought to be important for protecting mycobacteria from toxicity of reactive oxygen intermediates generated by host macrophages. Additionally, SOD is known to be a potent immunogen capable of eliciting antibody production and T-cell proliferative responses in animal models and in human tuberculosis patients.

MHC class I-restricted $CD8^+$ T-cell response during *M. tb.*-infection is not defined. We have utilized a matrix-based algorithm, EpiMatrix, to predict putative peptidic ligands derived from SOD of *M. tb.* for HLA-A0201 allele. Among three predicted peptides, one nonameric peptide was confirmed to be an HLA-A2-binder in the T2 stabilization assay. This SOD-derived peptide was then used to pulse peripheral blood mononuclear cells (PBMC) obtained from an $HLA\text{-}A2^+$ tuberculosis patient with lymphadenitis. PBMC cultures were re-stimulated with peptide-pulsed HLA-A2-expressing lymphoblastoid cells in the presence of IL-2. $CD8^+$ T cells were then isolated with anti-CD8 immunomagnetic beads and similarly re-stimulated. Thus, the $CD8^+$ cytotoxic T lymphocyte (CTL) line (98% $CD8^+$, 99% $CD3^+$) generated from PBMC of this patient recognizes a nonameric peptide derived from SOD of *M. tb.* The recognition is both HLA-A2-restricted and peptide-specific, as tested with a panel of 721.221-transfectants and known HLA-A2 ligands. Cytolysis of targets was inhibited by anti-MHC class I (W6/32) and anti-CD8 (C8) mAbs. Finally, a CTL bulk culture obtained from another $HLA\text{-}A2^+$ patient with active pulmonary tuberculosis recognized the same SOD-derived peptide. We propose that a nonameric SOD-derived peptide identified here represents the target CTL epitope for MHC class I-restricted $CD8^+$ T-cell response during *M. tb.*-infection.

CHARACTERIZATION OF A NEW VIRULENCE FACTOR IN PSEUDOMONAS AERUGINOSA INVOLVED IN BACTERIAL ATTACHMENT

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A novel in vivo pathogenesis system that utilizes a human clinical isolate of *P. aeruginosa* (strain UCBPP-PA14) infectious in both a mouse burn model and in a plant leaf infiltration model was developed by Rahme et al. (1) for the identification of virulence factors in *P. aeruginosa*. Screening of 2,500 transposon-generated mutants of *P. aeruginosa* strain PA14 for reduced virulence in plants led to the identification of previously unknown *P. aeruginosa* pathogenicity-related genes (2). One of these mutants, *33A9*, was chosen for further characterization because it also exhibited no mortality in a mouse burn model. DNA sequence analysis of the region that contains the *33A9* insertion showed no homology to other known virulence related genes. Protease, elastase and phospholipase activity assays as well as assays that measured the ability of the mutant to produce the secondary metabolite pyocyanin did not show any differences compared with the wild-type strain PA14, indicating that the mutation was not affecting these known pathogenicity factors. Further characterization of the *33A9* mutant showed that it was altered in attachment, an essential step in both bacterial virulence and biofilm formation. Attachment experiments done using polyvinylchoride (PVC) plates showed increased attachment for the mutant *33A9* compared to the wild-type PA14, and suggested a role for the *33A9* gene in the regulation of attachment to surfaces in a growth-phase dependent manner. Furthermore, motility experiments showed that the mutant *33A9* was affected in both pili and flagella-mediated motility, two elements that have been previously identified as necessary for bacterial attachment and biofilm development. Expression assays showed that transcript levels of *pilB* and *pilT* but not *pilA* were reduced in the mutant *33A9*. One hypothesis we are exploring is that the reduction in virulence observed for the mutant *33A9* in the mouse burn model could be explained by the increased attachment of the *33A9* mutant and its inability to disperse during host colonization. 1. Rahme, LG; Stevens, EJ; Wolfort, SF; Shao, J; Tompkins, RG and Ausubel, FM. 1995. Science 268: 1899-1902. 2. Rahme, LG; Tan, MW; Le, L; Wong, SM; Tompkins, RG; Calderwood, SB and Ausubel, FM. 1997. Proc. Natl. Acad. Sci. 94: 13245-13250.

M. TUBERCULOSIS GENES INDUCED DURING INFECTION OF MICE OR HUMAN MACROPHAGES: IDENTIFICATION BY IVET AND QUANTIFICATION OF GENE EXPRESSION BY RT-PCR

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We describe a new IVET system for selection of *in vivo* expressed promoters of *M. tuberculosis* and a novel RT-PCR system for quantitative analysis of gene expression. We have identified several genes of *M. tuberculosis* which are induced during infection of mice or human macrophages. Our IVET system is based upon expression of *inhA*, encoding enoyl-ACP reductase, which is involved in mycolic acid biosynthesis and is a major target for isoniazid (INH) in *M. tuberculosis*. Expression of *inhA*, under the control of promoters from *M. tuberculosis*, was used to select clones surviving INH treatment during the infection of either mice or human alveolar macrophages. We compared the levels of mRNA from the genes controlled by these promoters in cultures of *M. tuberculosis* growing in broth or in macrophages, using RT-PCR combined with molecular beacons and identified two promoters which were expressed at higher levels during infection of macrophages. One is the promoter for Rv0288, an ESAT6-like gene, and the other for a gene of unknown function, Rv2520c. Another promoter identified by the IVET system is that for two nearly identical genes, Rv1148c and Rv1945, of unknown function, which are members of the REP13E12 gene family.

***Salmonella enteritidis* infections of chicks.**

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Salmonella enteritidis, a leading cause of food poisoning in the US, is usually acquired from poultry products. Unlike other *Salmonella* serovars, S. *enteritidis* is able to colonize the oviduct of laying hens and infect the egg. *S. enteritidis* infects the devloping chick and the cycle of infection is completed. We have studied the development of *S. enteritidis* in day old chicks and defined the spread of *Salmonella* through the bird. This quantitative study will allow us to perform a detailed analysis of the effect of defined *S. enteritidis* mutants on disease formation in young chicks.

GENETIC ANALYSIS OF AN EXTRACELLULAR LIPASE OF *MORAXELLA BOVIS*

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Infectious keratoconjunctivitis is an economically important disease of cattle caused by the Gram-negative coccobacillus, *Moraxella bovis*. A number of factors contribute to the virulence of the organism but the two most important attributes so far identified are the presence of pili, and the ability to produce haemolysin. Other hydrolytic enzymes produced by *M. bovis* degrade lipids, proteoglycans and matrix proteins, and may be essential for the progression of the disease. This work describes the cloning and characterisation of an extracellular lipase. A lipolytic activity was associated with a 2.2 kb chromosomal DNA fragment expressed in *E. coli*. Nucleotide sequencing of the fragment revealed a 1.8 kb open reading frame capable of encoding a mature protein of 65.8 kD MW with DNA and protein similarity to the GDSL family of lipolytic enzymes. SDS-PAGE and N-terminal sequencing analysis of the culture supernatant from the recombinant *E. coli* revealed that the protein was abundantly secreted into the supernatant. Thin layer chromatography studies revealed that the lipase had a substrate degradation profile consistent with phospholipase B. The recombinant protein appeared to be toxic for animals but did not cause obvious cytopathic effects on bovine corneal epithelium and displayed unexpected heat stability properties.

A RESPONSE REGULATOR THAT REPRESSES TRANSCRIPTION OF SEVERAL VIRULENCE OPERONS IN THE GROUP A STREPTOCOCCUS

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A search for homologs of the *Bacillus subtilis* PhoP response regulator in the GAS genome revealed three good candidates. Inactivation of one of these, recently identified as *csrR* (Levin and Wessels, Molecular Microbiology. 1998. 30:209-219), caused the strain to produce mucoid colonies and increased transcription of *hasA*, the first gene in the operon for capsule synthesis. We report here that a non-polar insertion in this gene also increased transcription of *ska* (encoding streptokinase), *sagA* (streptolysin S), and *mf* (mitogenic factor) but did not affect transcription of *slo* (streptolysin O), *mga* (multiple gene regulator of GAS), *emm* (M protein), *scpA* (complement C5a peptidase), or *speB* or *speC* (pyrogenic exotoxins B and C). The amounts of streptokinase, streptolysin S and capsule paralleled the levels of transcription of their genes in all cases. Because CsrR represses genes unrelated to those for capsule synthesis, and because CsrA-CsrB is a global regulatory system in *E. coli*, the locus has been renamed *covR* for "control of virulence genes" in GAS. Transcription of the *covR* operon was also increased in the nonpolar insertion mutant, indicating that CovR represses its own synthesis as well. All phenotypes of the non-polar insertion mutant were complemented by the *covR* gene on a plasmid. CovR acts on operons expressed both in exponential and in stationary phase, demonstrating that the CovR-CovS pathway is separate from growth phase-dependent regulation in the GAS. Therefore, CovR represents the first multiple gene repressor of virulence factors described in this important human pathogen.

COMPARATIVE COMPLEMENT-MEDIATED KILLING AND COMPLEMENT DEPOSITION BETWEEN SMOOTH AND ROUGH MUTANT *Brucella abortus* AND *Brucella melitensis* STRAINS

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The brucellae are gram-negative intracellular pathogens that survive and multiply within phagocytic cells of their hosts. Smooth organisms present O-chain on their surface which is recognized by serologic tests and helps the bacteria avoid the bactericidal action of serum. Opsonization of smooth brucellae by treatment with normal human serum, however, enhances ingestion by macrophages. Rough strains lack surface O-chains, are more sensitive to serum-mediated killing, and have reduced virulence. The *wboA* gene, coding for the enzyme glycosyl transferase, is essential for the assembly of O-chain in *Brucella*. In this study, the sensitivity to serum of smooth, virulent *B. melitensis* 16M and *B. abortus* 2308, as well as rough mutant *Brucella* strains was assayed using normal non-immune human serum (NHS) at different concentrations and incubation times. Additionally, deposition of complement components (C1q, C2, C4, iC3b and C5b-9) and mannose binding protein (MBP) on the bacterial surface was detected using flow cytometric analysis. Rough strains RB51 and VTRA1 are *wboA* mutants derived from *B. abortus* 2308. RB51 contains at least one additional mutation. VTRM1 was derived from *B. melitensis* 16M by transposon (Tn5) disruption of the *wboA* gene. WRR51 was derived from *B. melitensis* 16M by replacement of the internal region of the *wboA* gene with an antibiotic resistance cassette. Rough *B. abortus* mutants RB51 and VTRA1 were more sensitive to the bactericidal action of NHS than rough *B. melitensis* strains VTRM1 and WRR51. Smooth Brucella strains 16M and 2308 deposited lower amounts of complement components at a slower rate compared to rough strains. Deposition of iC3b and C5b-9 and bacterial killing occurred when bacteria were treated with C1q-depleted serum, but did not occur when C2-depleted serum was used. Inclusion of Mg-EGTA during treatmenf of bacteria with NHS abolished both bactericidal activity and deposition of complement components. These results indicate that 1) OPS-deficient strains derived from *B. melitensis* 16M are more resistant to the bactericidal action of NHS than OPS-deficient strains derived from *B. abortus* 2308; 2) both the classical and the MBP-mediated pathways are involved in complement deposition and complement-mediated killing of *Brucella* strains; and 3) the alternative pathway is not activated by smooth or rough brucellae. Smooth brucellae may limit complement deposition on their surface to protect them from extracellular killing but allow sufficient deposition to opsonize them for uptake by macrophages, their preferred target for intracellular replication.

IDENTIFICATION OF A GENE WITHIN A PATHOGENICITY ISLAND OF ETEC H10407 REQUIRED FOR MAXIMAL SECRETION OF THE HEAT LABILE ENTEROTOXIN

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Studies of the pathogenesis of enterotoxigenic *E. coli* (ETEC) have largely centered on extrachromosomal determinants of virulence, in particular the plasmid-encoded heat-labile (LT) and heat stable (ST) enterotoxins, and the colonization factor antigens (CFAs). However, Tia, a putative adhesin of ETEC H10407, resides on a large chromosomal element of approximately 46 kb that shares multiple features with previously described *E. coli* pathogenicity islands (pais). Analysis of the DNA sequence downstream from *tia* revealed at least 4 closely spaced candidate open reading frames in the same transcriptional direction as *tia*. Comparison of the translations of these ORFs by BLAST-P revealed no significant homology to known virulence factors; although the predicted products of three of these ORFs were very similar to translations of uncharacterized ORFs (HP0731-733) in genome of *Helicobacter pylori*. Analysis of this same region revealed the presence of multiple potential motifs common to bacterial secretion systems. These include ATP-binding motifs, and an element found in EpsE, part of the general secretion pathway for cholera toxin. An in-frame deletion of one candidate gene within the island designated *leo*A (*l*abile *e*nterotoxin *o*utput), a close homologue of HP0731, resulted in a marked diminution of LT in culture supernatants, and the accumulation of LT in the periplasm. In the rabbit ileal loop model, this mutant strain (242.?4) promoted significantly less fluid accumulation than the H10407 parent. Complementation of 242.?4 with *leoA* in *trans* on the expression plasmid pES011 restored LT secretion into the supernatants to wild type levels. In addition, we demonstrate that LT secretion by EDL903, an LT^+ strain lacking the pai, is poor compared to H10407, a strain originally isolated from a patient with severe, cholera-like diarrhea. Although previous studies have suggested that *E. coli* lack the capacity to secrete LT, our studies suggest that maximal release of LT from the periplasm of H10407 is dependent on one or more elements encoded on a pathogenicity island.

ELUCIDATION OF THE MECHANISM FOR THE INTERACTION OF YOPD AND LCRH FROM *YERSINIA PSEUDOTUBERCULOSIS.*

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Pathogenic *Yersinia* spp. encode numerous virulence associated proteins on a large plasmid that incorporate a type III secretion mediated strategy enabling bacteria to remain extracellular during host infection. During host cell contact, bacteria actively translocate effector proteins into the cytosol of a target host cell. These cytosolic localised proteins subvert host cell inter- and intra-signaling pathways that inhibit bacterial phagocytosis and cellular recruitment. To this end, some proteins exhibit multiple functions acting both inside and outside the bacterium. For example, YopD (for *Yersinia* Outer Protein D) is essential for both control of Yop synthesis and for Yop-effector translocation into target cells. Moreover, a C-terminal potential amphipathic domain plays an important role in these processes. In addition, non secreted LcrH forms a putative complex with YopD in the bacterial cytoplasm that appears necessary for YopD stabilisation prior to secretion. Thus, it is conceiveable that the YopD/LcrH interaction is a significant event for coordination of YopD function. Based on this premise, we have focused on elucidating the mechanism of this interaction using a genetic approach as a preliminary tool to investigating YopD function in detail. Using the yeast two-hybrid system, we report herein that the putative internal membrane spanning and the C-terminal amphipathic domains of YopD are essential for permitting interaction with LcrH. Introduction of amino acid substitutions within the hydrophobic side of the amphipathic α-helix abolished the YopD/LcrH interaction, indicating that hydrophobic, as opposed to electrostatic forces of attraction are important for this process. Suppresser mutations isolated within LcrH correlate with this observation. The YopD and LcrH mutants generated are likely to be relevant as tools to understand YopD function during a *Yersinia* infection.

THE ROLE OF THE ADP-RIBOSYLTRANSFERASE ACTIVITY OF *PSEUDOMONAS* EXOENZYME S IN ITS CELLULAR MECHANISM OF ACTION

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Exoenzyme S (ExoS) is an ADP-ribosylating toxin that contributes to the virulence of the opportunistic pathogen, *Pseudomonas aeruginosa*. ExoS is translocated into eukaryotic cells via the type III secretory process where it exerts complex and cell- type dependent alterations in cell function which include the inhibition of DNA synthesis, altered cytoskeletal structure and interference of focal re-adhesion. Although the precise cellular mechanism of action of ExoS remains unknown, the amino-terminal domain is known to contribute to cytoskeletal alterations, while the carboxy-terminal domain includes the ADP-ribosyltransferase (ADPRT) activity of ExoS. To clarify the role of ExoS ADPRT activity in the cellular mechanism of action of ExoS, wild-type (WT) or enzymatically inactive (E381A) forms of ExoS were translocated into epithelial and fibroblastic cells using a derivative of *P. aeruginosa* strain PA103 which lacks production of other type III effector proteins. Inhibition of DNA synthesis and long-term cytoskeletal alterations were more pronounced in cells co-cultured with WT ExoS producing bacteria when compared with bacteria producing the E381A mutant form of ExoS, confirming that these effects on cell function resulted from the ADP-ribosylation of cellular proteins. We have previously identified the low molecular mass G (LMMG)-protein, Ras, as an in vivo target of ExoS ADPRT activity. Ras mediates the transduction of cell signals affecting proliferation, cytoskeletal structure and focal re-adhesion. Thus, alteration of Ras function by ExoS provides a potential cellular mechanism for observed effects of ExoS on cell function. More recently, however, we have also identified the LMMG-protein, Ral, as another in vivo target of ExoS ADPRT activity. Although the role of Ral in cell function is unclear, Ral GDS and Ral synergize with Ras in cellular transformation. We conclude from these studies that long-term cytoskeletal alterations and inhibitory effects of ExoS on DNA synthesis are mediated by ExoS ADPRT activity. The ability of ExoS to ADP-ribosylate two and possibly more cellular proteins following bacterial translocation also supports the hypothesis that effects of ExoS on cell function result from the coordinated modification of multiple cellular proteins, and that different patterns of substrate modification by ExoS may contribute to cell-type dependent alterations in cell function by ExoS.

A REGULATORY CASCADE INVOLVES INTEEGRATION HOST FACTOR (IHF) AND A LEE ENCODED REGULATORY PROTEIN (LER) CONTROLS THE EXPRESSION OF TYPE III SECRETION GENES OF ENTEROPATHOGENIC ESCHRICHIA COLI (EPEC)

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Enteropathogenic *E. coli* (EPEC) elicit changes in host cell morphology and cause actin rearrangement, a phenotype that has been commonly referred to as attaching/effacing (AE) lesions. The ability of EPEC to induce AE lesions is dependent upon a type III protein secretion/translocation system. The genes that encode this system are clustered in a 35.6-kb DNA segment, named the locus of enterocyte effacement (LEE). Given that the LEE is a horizontally acquired DNA, it is expected that it will contain elements that act in concert with the general chromosomal regulatory circuits of the *E. coli* host.

We initiated this study to identify global regulators that are needed for the expression of LEE genes. Using transcriptional fusions between LEE genes and the green fluorescence protein (*gfp*) reporter gene, we found that the DNA binding protein integration host factor (IHF) is essential for the expression of the LEE genes encoding type III secretion components and effecor molecules. Yet, IHF activates directly only a relatively small number of LEE genes, among these is the *ler* gene. The *ler* encoded Ler protein functions as a global regulator that mediates the expression of numerous LEE genes. Formation of AE lesions and EPEC invasion is dependent on both IHF and Ler. Thus IHF and Ler form a complex regulatory cascade necessary for the formation and assembly of functional type III secretion system.

PHENOTYPIC SWITCHING OF THE *CRYPTOCOCCUS NEOFORMANS* VARIANT RC-2 OF STRAIN 24067 *IN VITRO* AND *IN VIVO*.

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Here we investigated whether the virulent *C. neoformans* strain RC-2 can undergo phenotypic switching *in vitro* and *in vivo*. We present data showing RC-2 switches *in vitro* from a smooth (SM) standard colony type to a mucoid (MC) colony type at a frequency of 0.5-1 x 10^{-4}. The switch to a MC phenotype changes cell size (SM: 7.4 $\pm$ 1.9 versus MC: 9.9 $\pm$ .67, $p<.001$) and capsule size (SM 1.05 $\pm$ 0.15 versus MC 2.2 $\pm$ 0,25, $p<0.001$). The *in vitro* doubling time differs minimally for SM and MC and is 2.4 h versus 2.8 h at 30^{0}C and 2.2 h versus 2.7 h at 37^{0}C. The chemical structure of glucoronoxylomannan (GXM), the main component of the polysaccharide, was identical for SM and MC. Virulence was studied in an intra tracheal infection model in A/J mice and these experiments showed that MC is significantly more virulent than SM (mean survival was 23.3 d for MC andConfidential Page 108/31/99 42.3 d for SM, $p< .0001$). The increase in virulence was associated with decrease in inflammatory response and a increase in fungal tissue burden and lung weight.(log CFU/ total lung 4.7 $\pm$ 0.61 for SM versus 5.9 $\pm$ 0.66 for MC, $p=0.013$). Mice injected with SM phenotype exhibited MC colonies in lung tissue at a rate of 0.1-3.1% which represents a 10 - 300 fold increase compared to *in vitro* switching. There are several differences between the RC-2 switching system and previously described switching systems, where *C. neoformans* was shown to switch from a SM to a wrinkled (WR) colony morphology. First, the MC phenotype represents a frequently occurring phenotype in contrast to WR colony phenotypes, which are rarely observed in environmental and clinical *C. neoformans* isolates. Second, the MC phenotype can be recovered from mice injected with SM phenotypes suggesting that phenotypic switching occurs *in vivo* and is selected for by the immune system.

PSEUDOMONAS AERUGINOSA TYPE III SECRETED EFFECTORS THAT INFLUENCE CYTOTOXICITY AND INVASION

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Pseudomonas aeruginosa is a major cause of hospital-acquired pneumonia, a leading cause of sepsis and death in immunocomprimised patients and those afflicted with cystic fibrosis. Like several other gram negative pathogens, *P. aeruginosa* expresses a type III secretion system which is required for virulence. Thus far four type III secreted effectors have been identified for *P. aeruginosa*: ExoS, ExoT, ExoU and ExoY. ExoS and ExoT are closely related ADP-ribosyltransferases, ExoY is an adenylate cyclase, and ExoU is an acute cytotoxin of unknown mechanism. Our work has mainly focused on the *P. aeruginosa* clinical isolate PA103 which expresses ExoT and ExoU, but not ExoS or ExoY. PA103 is cytotoxic toward and does not invade epithelial cells (MDCK) or macrophages (J774) in vitro. In contrast, type III secretion mutants of PA103 are non-cytotoxic toward and are internalized by these cell types. PA103*exoU* mutants (which do not express the known acute cytotoxin ExoU, but are still capable of type III secretion) are non-invasive, non-cytotoxic toward epithelial cells, yet continue to show appreciable cytotoxicity toward macrophages. These results prompted the hypothesis that in addition to known effectors PA103 delivers both an anti-internalization factor and an additional cytotoxin by type III secretion. To identify these factors we are conducting a genetic screen. A transposon library has been constructed in a PA103*exoU* background. Individual clones from this library are being screened using an aminoglycoside exclusion assay for increased internalization by macrophages due to loss of expression of the anti-internalization factor. Simultaneously, clones are screened for loss of cytotoxicity toward macrophages by evaluating Trypan Blue Stain exclusion by macrophages in infected culture wells. Thus far we have identified four classes of mutants: clones which pass both screens that are likely to harbor defects in type III secretion itself; clones which are non-invasive, non-cytotoxic and fail to display twitching motility that are likely to be defective in pili expression: clones which are non-invasive, non-cytotoxic and display twitching motility that may have a mutation in the unknown cytotoxin; and finally clones which are invasive and non-cytotoxic that may have mutations in the putative anti-internalization factor. We are continuing to screen this library and are further characterizing the phenotypes of these clones. The identification of the affected genes in these clones may reveal novel type III secreted effectors important to the virulence of *P. aeruginosa*. We have also conducted preliminary studies using PA103*exoT* and PA103*exoU/exoT* mutants that suggest that ExoT may have anti-internalization activity toward MDCK cells, but does not contribute to cytotoxicity toward macrophages.

IMMUNORESPONSE TO TYPE III-SECRETED PROTEINS OF *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS PATIENTS

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Patients with the hereditary disease Cystic Fibrosis (CF) often suffer from chronic infections with *P. aeruginosa*. Early detection of this bacterium could lead to fast antibiotic intervention and repression of chronic colonization during the first years of life, but detection and culture are often difficult in early childhood.
Similar to *Yersinia enterocolitica P. aeruginosa* uses a so-called type III-secretion-system to translocate proteins into the cytosol of eukaryotic cells, where these proteins interfere with signal transduction pathways and other important cell functions.
For *P. aeruginosa* several type III-proteins are described until now, as e. g. PcrV, ExoS, ExoT, ExoU and ExoY.
In *Y. enterocolitica* type III-secreted proteins und proteins encoding the secretion machinery are useful antigens for serological diagnosis of *Yersinia* infections. Therefore we prepared recombinant type III-proteins of *P. aeruginosa* and tested sera of CF-Patients for antibody-reactivity.
We correlated these results with *P.aeruginosa*-colonization in these patients.
We found that all patients with chronic *Pseudomonas* colonization had antibodies against PcrV while antibodies against other type III-proteins were inconsistently expressed. Corresponding *Pseudomonas* strains were tested for genotypical and phenotypical presence or absence of type III-proteins. As for the high homology between some *Yersinia* and *Pseudomonas* type III-proteins, crossreactivity between these proteins in *Y. enterocolitica* and *P. aeruginosa* was examined.

ANALYSIS OF THE EXPRESSION OF IDER-REGULATED GENES IN *MYCOBACTERIUM TUBERCULOSIS* (*MTB*) DURING IRON LIMITATION AND *MTB* INFECTION OF HUMAN MACROPHAGES

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In this work we characterize the regulon of IdeR (Iron dependent repressor), the major repressor of *Mtb* iron acquisition. IdeR binds a 19 bp palindromic sequence termed an "iron box" located at the -10 or -35 region in the promoter of an IdeR-regulated gene. Using the programs "findpatterns" in GCG and "pattern search" in the Tuberculist, we probed the published *Mtb* genome for the IdeR iron box sequence. Genes preceded by iron boxes included a set encoding proteins directly involved in iron acquisition, such as the biosynthesis of siderophores (*mbtB*, *mbtA*, *mbtI*) and aromatic amino acids (*pheA*, *hisE*, *hisB*-like), iron-uptake (an ABC transporter with similarity to the yersiniabactin uptake machinery), and iron-storage (*bfrA*, *bfrB*). Some putative IdeR-regulated genes encoded proteins with no known relationship to iron and were similar to genes involved in the biosynthesis of LPS, lipids (*acpP*) and peptidoglycan (*murB*). To validate our predicted iron-box sequences, we analyzed the iron-dependent expression of four genes, *mbtB*, *mbtI*, Rv3402c (similar to LPS, perosamine or 3-amino-5-hydroxybenzoic acid biosynthesis) and *bfrA* (bacterioferritin). The mRNA levels of *mbtB*, *mbtI* and Rv3402c were induced 28, 29, and 18-fold, respectively, in cultures of *Mtb* grown in low iron compared with high iron, and expression of these genes was derepressed in the *Mtb ideR*⁻ null mutant. The mRNA levels of these three genes also increased during *Mtb* infection of macrophages. In contrast, the *bfrA* mRNA levels decreased when *Mtb* were grown in low iron or during macrophage infection. These data suggest the mycobacterial phagosome is limiting for iron. In addition, IdeR, in addition to its role as a repressor of transcription, may act as an activator. Gel-retardation experiments and DNase footprinting analyses with purified IdeR show that IdeR binds to these iron boxes *in vitro*.

BACTERIAL FACTORS INDUCED DURING A SYSTEMIC *Y. ENTEROCOLITICA* INFECTION

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Yersinia enterocolitica is a Gram-negative pathogen that causes numerous disease states upon infection of humans. Most commonly, infection results in a self-limiting enteritis. However, upon infection of immunocompromised persons *Y. enterocolitica* can gain access to the blood, spleen, liver and lungs resulting in a systemic infection. Systemic infections are characterized by a striking 50% mortality rate.

An enrichment for *Y. enterocolitica* genes that are active during a systemic infection was developed. A library of promoters fused to a promoterless *cat* gene was used to infect BALB/c mice and chloramphenicol was administered. If the promoter is active the expression of *cat* will render the bacteria resistant to chloramphenicol. Those promoter fusions that were preferentially expressed during infections of BALB/c mice were cloned and sequenced. Thirty different alleles have been identified and given a *sif* (for systemic infection *f*actor) designation. The proposed physiological functions of the *sif* alleles, based on sequence homology, include O-antigen and fatty acid biosynthesis, transporters, regulatory proteins, as well as unknown functions.

Two alleles, *sif1* and *sif2*, have been further characterized. *sif1* shows sequence homology to a component of ABC transporters. *sif2* is in a region of the chromosome homologous to the *lep* operon of *E. coli*, but the function is unknown. *In vivo* expression analysis, using the *cat* gene fusions, confirmed that both alleles are active during systemic infections of BALB/c mice. Fusions to the reporter gene, *lacZ*, have been used to identify laboratory conditions that affect expression. Very little *sif1* expression has been measured under all conditions tested thus far, suggesting its expression may be limited to infection conditions. In contrast, *sif2* expression has been shown to be induced in 1% tryptone media and during stationary phase. Mutants of *sif1* and *sif2* were used to infect BALB/c mice to determine if the infection kinetics were altered compared to wild-type *Y. enterocolitca*. In addition, cosmids containing the wild-type alleles were sequenced and analyzed to determine the chromosomal organization of these genes.

Bacillus anthracis germination: an intracellular step for an extracellular pathogen

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The fatal character of the infection caused by inhalation of *Bacillus anthracis* spores results from a complex pathogenic cycle involving the transformation of a dormant spore into a vegetative cell. This step enables bacteria to actively proliferate and to synthesize their virulence factors such as toxins. We have shown, using immunofluorescent staining, confocal scanning laser microscopy and image cytometry analysis that the alveolar macrophage is the primary site of *B.anthracis* germination in a murine inhalation infection model. Germination occured in endocytic vesicles derived from the phagosomal compartment. We have also demonstrated that the toxin genes were expressed within the macrophages following the germination. We identified a tricistronic operon of germination (*gerX*) on the virulence plasmid pXO1. The three predicted proteins have significant sequence similarities to *B. subtilis*, *B.cereus* and *B.megaterium* germination proteins. Expression of the *gerX* operon was detected exclusively in the forespore compartment. A *gerX-null* mutant was less virulent than the parental strain and did not germinate efficiently *in vivo* or *in vitro* within phagocytic cells. These data strongly suggest that *gerX*-encoded proteins are involved in the virulence of *B.anthracis*.

IDENTIFICATION OF SALMONELLA LOCI NECESSARY FOR RESISTANCE TO BILE

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Bile salts are detergents made by the liver that are stored in high concentrations in the gallbladder and released into the intestine to aid digestion. Salmonella spp. come in contact with bile salts in the intestine, and in the chronic carrier state, within the gallbladder. Salmonella is able to resist the action of bile and respond to escalating bile concentrations by increasing mechanisms of resistance. The PhoPQ two-component regulon is necessary for high-level bile resistance, and we invoked a transposon mutagenesis screen to identify PhoPQ-dependent and PhoPQ-independent mechanisms of bile resistance. A PhoP-constitutive strain was mutageneized followed by screening for the loss of bile resistance on agar plates. From the screening of 11,000 colonies, 15 bile-sensitive strains were identified. Of these, five were not characterized further because of slow growth, rough LPS, or because they possessed identical insertions as determined by Southern blot experiments. None of the remaining eight unique bile sensitive insertions were in *phoPQ*, *mar* or *acr* loci, which are known to affect resistance to bile. The minimal inhibitory concentration for bile, specific bile salts, SDS and Triton-X-100 was defined for each strain, which showed that all were susceptible to one or more bile salts or detergents (range; 2- to 483-fold). The transposon/chromosomal DNA junctions of all eight strains was cloned and sequenced, which identified three insertions within or linked to *tolQ*, and other insertions in loci of unknown function. Bile and PhoPQ regulation of the MudJ trasposon-generated fusions was also examined. This work has provided us with increased knowledge of this relatively unknown area of host-bacterium interactions, and may provide insight into mechanisms of resistance and response to bile that will help us understand the development of the carrier state.

IDENTIFICATION AND CHARACTERIZATION OF *SALMONELLA* MUTANTS ALTERED FOR INTRACELLULAR LOCALIZATION AND REPLICATION

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The ability of *Salmonella typhimurium* to replicate within a membrane-bound vacuole while inside epithelial cells correlates strongly with its ability to cause disease. In an effort to identify additional virulence genes involved in intracellular pathogen-host interactions, two approaches have been taken. The first approach is based on a novel phenotype exhibited only by *Salmonella* replicating within epithelial cells This phenotype is characterized by the aggregation of host vacuoles into tubular compartments that interconnect with the *Salmonella* -containing vacuole. One gene required for the phenotype, *sifA*, is also required for full virulence; therefore, we have developed a direct, high-throughput screening method to identify *S. typhimurium* mutants unable to induce the formation of host-derived tubules. The use of 96-well Special Optics plates has made it feasible to screen 10,000 individual transposon mutants for a visual intracellular phenotype. The second approach utilized a cefotaxime enrichment assay to identify mutants that have a reduced ability to replicate inside the host vacuole. Three mutants previously identified by the cefotaxime enrichment assay (Leung et al., 1991 *PNAS* 88(24): 11470) have been further characterized and demonstrate the value of this method. Two of the mutants contained insertions in genes involved in DNA mismatch repair: *MutS* and *MutH*. The third mutant is disrupted in *ssaQ*, one of thirteen structural genes of the type III secretion system apparatus encoded within *Salmonella* Pathogenicity Island 2 (SPI-2). This is the first study to implicate a role for DNA repair mechanisms in intracellular replication and suggests that SPI-2 effectors may also have a role in creating a replication-permissive vacuole. Characterization of recently obtained mutants in host vacuole aggregation and intracellular replication is ongoing and will be presented.

DRUG TARGET EVALUATION THROUGH CONDITIONAL ALLELES

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We have developed a rapid method for the production of artificial conditional alleles in the yeast *Saccharomyces cerevisiae*. The strategy allows production of artificial conditional alleles of virtually any known gene or open reading frame, facilitating rapid depletion of the specific gene product. This serves as a potent model of specific inactivation of the gene of interest by an idealized drug. Our method is an extension of the copper sensitization strategy first developed by Zarmik Moqtaderi and Kevin Struhl. In this system, the ROX1 and UBR1 genes are placed under the control of a copper-inducible promoter. The gene of interest is placed under the control of the ANB1 promoter. In the absence of exogenously added copper, neither the ROX1 repressor nor the UBR1-dependent degradation system is active, so the protein of interest is produced. In the presence of copper ions in the media, transcription of the gene of interest is repressed by the ROX1 gene product, and existing protein is depleted by the ubiquitin degradation system. We have improved the system to make it more robust, and to increase the rapidity with which we can create strains. Because very high levels of copper are known to be toxic to yeast, we have chosen a strain of *Saccharomyces* which has a high tolerance for copper ions in the media. The SLF1 gene, which has been shown to have an essential role in the biomineralization of copper, has been deleted from this strain. We have developed a rapid PCR approach for producing plasmids which can be used to put the genes of interest under the control of the system, and we have designed the constructs so that the resultant recombinant strains do not require selection in order to stably maintain the engineered changes. To date we have constructed and tested over 300 strains using this system. In addition to efficiently assaying essentiality, the system can be used to assay the effects of specific gene product depletions on the viability of cells. The cidality associated with gene product depletion varies widely, from the extreme of CDC15, an essential cell cycle checkpoint gene whose depletion even over long periods of time (>24 hours) has no fungicidal effect, to genes whose depletion leads to cidality of three or more orders of magnitude in a few hours. In addition to serving as a model for anti-fungal drug discovery, the method should prove adaptable for studying coordinated down-regulation of multiple genes.

A HUMAN MONOCLONAL ANTIBODY (98-6) REACTS WITH THE FUSION ACTIVE FORM OF GP41

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A mixture of two peptides from gp41 (N36 and C34) forms an ahelical structure that is thought to represent the fusion active core of gp41. A human monoclonal anti-gp41 antibody (mAb 98-6), generated from the cells of an infected individual reacted poorly with C34, but binding was strongly enhanced when N36 was added, indicating that the mAb reacts with a conformational epitope present in the fusion active core structure formed by the interaction of peptides N36 and C34. The epitope recognized by mAb 98-6 was found in virions on oligomeric forms of gp41 (dimers, trimers and tetramers). On infected cells the epitope was present as oligomers of gp41, as monomers of gp41, and as part of the envelope polyprotein gp160. In infected cells, the epitope was present as part of both monomeric gp41 and gp160. These studies demonstrate that infected humans can respond to the fusion active form of gp41 and that the anti-gp41 mAb studied here recognizes a conformational epitope formed by the interaction of two regions of gp41 which form an a-helical bundle. This epitope is found on several forms of gp41 as they occur in virions, on the surface of infected cells, and in infected cells.

MONITORING OF *SALMONELLA* VIRULENCE GENE EXPRESSION BY USE OF THE GFP REPORTER IN TARGETTED ORGANS DURING MURINE INFECTION

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Regulation of virulence gene expression in *Salmonella typhimurium* involves responses to a variety of environmental factors. Some *Salmonella* virulence gene products are specifically involved in the invasion of the ileal epithelium of the gastrointestinal tract whereas others are required for systemic spread and intracellular survival of the pathogen. However, up to now it has been difficult to determine whether virulence genes exhibit organ-specific or cell-type-specific patterns of expression.

We have now developed an improved system for assessing the transcriptional response of *Salmonella* virulence genes during either murine infection or invasion of epithelial cells. Based on the Green Fluorescent Protein (GFP) from *Aequorea* jellyfish, this system allows the monitoring of the expression of SPI1, SPI2 and other virulence genes within specific organs or cultivated cell-lines. GFP fluorescence in individual bacteria is visualised and quantified by flow cytometric and fluorescent microscopic techniques.

Preliminary experiments performed with the *S. typhimurium* strain 12023 harbouring plasmid-borne *gfp* transcriptional fusions to SPI2 genes will be described. These involved the measurement of gene expression in spleen and liver following murine infection and in epithelial cells. We will discuss the utility of this approach for monitoring transient induction of virulence genes during infection.

ANTIBODIES IN SERA FROM ETEC PATIENTS AND VACCINEES AGAINST LINEAR EPITOPES IN ETEC COLONIZATION FACTOR ANTIGEN I

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Human enterotoxigenic *Escherichia coli* (ETEC) cause diarrhea by colonization of the intestine by means of antigenically distinct colonization factors (CFs, ☮20 have been identified to date) and subsequent production of enterotoxin(s). Several of these CFs have significant amino acid (aa) sequence similarity, particularly in the N-terminal end. We have attempted to define linear B-cell epitopes in the fimbrial subunit of CF antigen I (CFA/I), a member of a sequence homology family, since such epitopes may be utilized in the development of a cross protective CF-peptide vaccine.

Sera drawn from Bangladeshi children and adults, naturally infected with CFA/I-expressing ETEC, as well as adult Swedes vaccinated with an oral inactivated CF-containing ETEC vaccine were analyzed in Pepscan for reactivity with overlapping hexamer peptides of the CFA/I fimbrial subunit protein (aa 1-52, aa 85-114, and aa 121-147). In all instances were six linear epitopes identified comprising aa 4-11, 13-20, 30-38, 39-48, 98-108, and 130-140, respectively. The number of epitopes identified and their sequences were identical irrespective of whether the sera were collected from infected Bangladeshi children and adults or from vaccinated adult Swedes. Sera drawn repeatedly from a Bangladeshi child after natural infection was also tested and irrespective of if the sera were collected early or late after infection the same six epitopes were identified. Furthermore, sera from a Bangladeshi child naturally infected with a CS14-expressing ETEC strain, belonging to the same sequence homology family as CFA/I, identified these same epitopes.

By comparing the aa sequences of the members of the CFA/I sequence family we could also show that three of the N-terminal linear epitopes identified are virtually identical in all members of the CFA/I-family, suggesting the existence of several cross-reactive epitopes.

tmRNA IS AN ESSENTIAL FUNCTION FOR *NEISSERIA GONORRHOEAE*

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Escherichia coli tmRNA, also known as 10Sa RNA, is a small abundant RNA that has both tRNA and mRNA properties. Homologues of tmRNA have been identified in every genus of bacteria examined. tmRNA is not an essential function in *E. coli*, nor has it been shown so in other bacteria. Keiler *et al.* proposed that the *E. coli* tmRNA, through both of its activities, targets truncated proteins for degradation by adding an 11 amino acid tag to their carboxyl termini. Studies from this laboratory have shown that, at least for the growth of some λ*imm*P22 hybrid phages, tagging for degradation is not likely to be the primary function of tmRNA.

We report studies on the tmRNA from *Neisseria gonorrhoeae*. Even though only sharing about 50% homology with the tmRNA of *E. coli*, the tmRNA of *N. gonorrhoeae* functions in *E. coli* to support the growth of a tester λ*imm*P22 hybrid phage. Derivatives of *N. gonorrhoeae* with an insertion disruption in the *ssrA* gene ($ssrA^{Ng}$), which encodes tmRNA, could not be constructed, suggesting that, unlike *E. coli*, *N. gonorrhoeae* requires tmRNA for viability. Studies with mutant $ssrA^{Ng}$ genes suggest that while this essential role includes the tagging activity of tmRNA, it does not include the associated protein degradation. Although derivatives of *N. gonorrhoeae* with disrupted $ssrA^{Ng}$ gene could be constructed from *ssrA* heterodiploid strains that also carry a copy of the *E. coli ssrA* ($ssrA^{Ec}$) gene, it appears that these derivatives grew only after acquiring some type of mutation(s). The nature of this putative suppressor mutation(s) is unknown. This and other results suggest a role in the action of tmRNA for regions other than those directly involved in the tagging process.

REDUCED IL-8 PROTEIN EXPRESSION WITH INCREASED IL-8 mRNA LEVEL IN *PORPHYROMONAS GINGIVALIS* INFECTED EPITHELIAL CELLS. G. T.-J. Huang, J. K.-H. Lee, H. K. Kuramitsu and S. Kinder Haake (UCLA School of Dentistry and SUNY Buffalo School of Dental Medicine, USA)

Interaction of bacteria with mucosal surfaces can modulate the production of proinflammatory cytokines produced by epithelial cells. *Porphyromonas gingivalis*, a key periodontal pathogen, adheres to and invades oral epithelial cells. Previously, we showed that interleukin-8 (IL-8) expression by gingival epithelial cells increases following interaction with several putative periodontal pathogens. In contrast, IL-8 expression is reduced after *P. gingivalis* ATCC 33277 challenge. In the current study, we investigated the kinetics of IL-8 expression in *P. gingivalis* infected gingival epithelial cells. IL-8 secretion was rapidly down-regulated within 2 h after infection, whereas IL-8 mRNA was up-regulated 2 to 4 h post-infection and then decreased to basal levels 8 to 20 h after *P. gingivalis* infection. Gingival epithelial cells were also challenged with several *P. gingivalis* strains including two strain 381 Arg-gingipain protease mutants, MT10 and G-102, which are defective in the *rgpA* and *rgpB* genes, respectively. Strain 381 as well as the two protease mutants also down-regulated IL-8 expression. In contrast, *P. gingivalis* W50 and W83 did not reduce IL-8 expression. *P. gingivalis* ATCC 33277, 381 and its mutants, G-102 and MT10, adhered/invaded gingival epithelial cells, whereas *P. gingivalis* W50 and W83 did not. Taken together, these results suggest that IL-8 attenuation appears to be associated with *P. gingivalis* attachment/invasion and is initially controlled at the post-translational level while the later stages of expression are controlled by transcriptional attenuation. The Arg-gingipain proteases are not individually involved in IL-8 attenuation. (Supported by UCLA Academic Senate Research Grant and UCLA School of Dentistry Research Opportunity Grant)

ROLE OF THE EXTRACYTOPLASMIC STRESS REESPONSE IN THE VIRULENCE OF *SALMONELLA TYPHIMURIUM*

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Vertebrates possess a range of non-specific defences that effectively prevent infection by most bacteria. Successful pathogens have evolved means to counteract or repair the damage inflicted by the host defences. The outer layers of bacteria interact directly with their host and consequently often bear the brunt of the assault from the hosts anti-microbial armament. In enteric gram negative bacteria damage to cellular components (particularly proteins) that resided outside of the cytoplasm is regulated by two stress response pathways. The first is controlled by the alternative sigma factor sigma factor E (σ^E, RpoE) and the second by the two component regulator CpxAR. Other than regulatory genes, the genes that are so far known to be positively regulated by σ^E and CpxAR are all enzymes involved in protein folding or degradation within the periplasm. One gene, *htrA*, is regulated by both σ^E and CpxAR. HtrA, a serine protease, is important for survival of salmonella within macrophages, resistance to reactive oxygen intermediates (ROI's) and virulence in mice. The role of other extracytoplasmic stress response (ESR) genes in the virulence of salmonella sp. has not been investigated. Recently, we showed that inactivation of *rpoE* highly attenuates *S.typhimurium*. The mutant was more sensitive to ROI's, antimicrobial peptides and killing by macrophages than either its wild type parent or an isogenic *htrA* mutant. The *rpoE* mutant was also less able to survive in the tissues of mice infected by both the oral and IV routes than a *htrA* mutant. Unlike *S.typhimurium htrA* mutants, the *S.typhimurium rpoE* mutant was poorly immunogenic. The *rpoE* mutant was not temperature sensitive, unlike its *E.coli* counterpart, but it did exhibit aberrant growth in liquid media lacking glucose. It also survived poorly in stationary phase, particularly in the absence of glucose. These results suggest that σ^E regulated genes other than *htrA* are important for salmonella virulence and growth. The only other (non-regulatory) gene controlled by σ^E is *fkpA* which encodes a periplasmic peptidyl prolyl isomerase. We have constructed and currently analyzing *S.typhimurium fkpA* and a *fkpA htrA* mutants. We are also investigating the role of the other ESR pathway on *S.typhimurium* virulence. To this end we have inactivated the *cpxR* gene of *S.typhimurium* and we are investigating the effect of this mutation on *S.typhimurium* physiology and virulence.

IDENTIFICATION OF VIRULENCE FACTORS IN *HISTOPLASMA CAPSULATUM* INVOLVED IN HOST CELL INTERACTION

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Histoplasma capsulatum is a dimorphic, pathogenic fungus that infects humans, causing pulmonary and systemic disease. It exists in mycelial form in the soil, and when aerosolized, the conidia are inhaled by the host. At 37°C, within the host, *H. capsulatum* undergoes a phase transition from mycelia to yeast and is phagocytosed by macrophages. This organism survives within the phagolysosome and is capable of inhibiting the acidification of that compartment. Recent advances in molecular and genetic tools in *H. capsulatum* now make it possible to identify genes involved in its pathogenesis.

Our goal is to use *Saccharomyces cerevisae* as a tool to identify genes from *H. capsulatum* involved in the interaction with host cells. *H. capsulatum* is capable of modulating the pH of the phagolysosome, and this modulation requires protein synthesis. In addition, it is also able to neutralize the pH of its media. We are expressing cDNAs from *H. capsulatum* in *S.cerevisiae* to identify genes that confer the ability to modulate the pH of the media. These genes will then be mutated in *H. capsulatum* to determine their role in pathogenesis and pH modulation of the phagolysosome.

H. capsulatum is also capable of infecting epithelial cells. *S.cerevisiae* expressing *H. capsulatum* genes will be used to identify genes involved in the adhesion of *H. capsulatum* to epithelial cells. Again, these genes will be mutated to determine their role in the pathogenesis of *H. capsulatum*.

THE PUTATIVE IRON TRANSPORT OPERON *SITABC* IS INVOLVED IN THE SYSTEMIC STAGES OF *SALMONELLA TYPHIMURIUM* INFECTION

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Salmonella typhimurium is an invasive pathogen that causes typhoid like disease in BALB/c mice. During the infection process the bacteria induce a number of gene products to evade host defenses and establish infection. These genes that are induced in response to the environment in the host can be directly selected using *in vivo* expression technology or IVET. Using IVET in cultured hepatocytes we have identified a putative iron transport operon in Salmonella Pathogenicity Island 1 (SPI1) designated *sitABC* (Zhou and Galan, 1999). SitABC shows high degree of homology to the *yfe* locus in *Yersinia pestis* and other ABC transporters in *Haemophilus influenzae* and *Synechocystis* spp. This operon is induced five fold *in vitro* under iron limiting conditions, and is repressed by the global regulator Fur in excess iron. In the animal, *sitABC* is induced specifically during the systemic stages of growth. The putative iron operon is also significantly attenuated for virulence in spleens and livers of BALB/c mice in competition assays with the wild-type. SitABC seems to belong to a subfamily of ABC transporters that are defined by their periplasmic binding proteins rather than their outer membrane receptor. All high affinity iron transport systems known thus far in *S. typhimurium* need TonB for their transport and it seems to be required during all stages of *Salmonella* infection. We are currently examining if the *sitABC* locus is dependent on the inner membrane protein TonB for providing energy for its substrate transport.

IDENTIFICATION OF A CYTOSOLIC ACTIVATOR OF THE *YERSINIA* OUTER PROTEIN (YOP) EFFECTOR YPKA

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The *Yersinia* outer protein (Yop) effector YpkA is a serine/threonine protein kinase translocated by the bacteria into host immune cells where it is hypothesized to thwart signal transduction cascade(s) via its kinase activity. YpkA recombinantly expressed in bacteria does not exhibit kinase activity in an *in vitro* kinase assay. However, using an *in vitro* kinase assay measuring autophosphorylation and phosphorylation of artificial substrates, we have identified a eukaryotic activator of YpkA kinase activity. Addition of crude extract from HeLa cells as well as rat tissue extracts (brain, spleen, liver, kidney) to this recombinantly expressed YpkA results in its activation. In addition, transient transfection of YpkA in HeLa cells results in an active form of YpkA. Subcellular fractionation of HeLa cell extract revealed the activator is located in the cytosol. Both boiling and trypsin treatment of the cytosol destroys this activation potential, suggesting the activator is proteinaceous. Furthermore, biochemical characterization using gel filtration suggests the activator is approximately 20 kDa. We are currently purifying this activator. The identification of this activator will not only broaden our understanding of the mechanism of activation of YpkA, but also contribute to our ability to identify the *in vivo* target(s) of YpkA.

ENTEROBACTERIAL NRAMPS: TRANSPORT, EXPRESSION AND PHENOTYPES

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Escherichia coli and *Salmonella typhimurium* each encode a single homolog of the Eukaryotic natural resistance-associated macrophage protein (NRAMP). We have cloned both homologs, characterized their transport, and identified some of their promoter regulation elements and associated phenotypes.

The Enterobacterial NRAMP, which we name MntA, is a highly selective transporter responsible for all known Manganese(II) flux in both organisms. $^{54}Mn^{2+}$ uptake is rapid and extensive. Incubation of cells at < 1 μM Mn^{2+} results in accumulation of ~50% of the total Mn^{2+} in less than 10 minutes with an internal concentration up to 10 mM and a transmembrane gradient up to 10^5:1. This is a dynamic steady state, as MntA also mediates Mn^{2+} efflux. The V_{max} for uptake is pH-sensitive, increasing 4-fold as the pH decreases from 8.2 to 5.5, consistent with the proton:divalent cation symport mechanism described for the mammalian NRAMP2 (rat DCT1). The K_m is insensitive to pH over this range, suggesting that $K_m \sim K_d$ and that Mn^{2+} binding does not involve any of the transmembrane histidines conserved in Eukaryotic and Bacterial NRAMPs.

Inhibition results suggest that Mn^{2+} is the only physiological substrate of MntA. Mn^{2+} uptake is inhibited strongly by Cd^{2+} (K_i < 1 μM) and growth sensitivity suggests Cd^{2+} may actually be transported. Fe^{2+} and Co^{2+} inhibit, but with K_i's of 10 to 30 μM at pH 5.5 their uptake is unlikely to be physiologically relevant. Ni^{2+}, Cu^{2+}, Zn^{2+} and Fe^{+3} inhibit weakly (K_i > 100 μM). In contrast to the K_m for Mn^{2+}, the K_i's of all these inhibitors are lower at pH 5.5 than at pH 8.2, implying that their binding differs from that of Mn^{2+} at its rate-limiting transport site

MntA is induced by several conditions encountered upon host infection. LacZ fusions in both *E. coli* and *S. typhimurium* LT2 are synergistically induced by cation starvation and hydrogen peroxide, consistent with the presence of potential Fur and OxyR binding motifs in the *mntA* promoter. An *mntA:lacZ* fusion in *S. typhimurium* SL1344 is also expressed upon infection of the murine macrophage cell line RAW264.7.

Overexpression of MntA makes both *E. coli* and *S. typhimurium* more sensitive to Mn^{2+} and Cd^{2+} than wild type cells, consistent with the very low basal Mn^{2+} transport levels of uninduced cells. Inactivation of chromosomal *mntA* renders free living bacteria more sensitive to external hydrogen peroxide; it does not markedly affect the ability of *S. typhimurium* SL1344 to invade and replicate in HeLa cells.

IMMUNE RESPONSE TO LIPOPOLYSACCHARIDE O-ANTIGEN OF *SALMONELLA TYPHIMURIUM*

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Lipopolysaccharide (LPS) forms the outermost layer of gram-negative bacteria and serves as a barrier between the cell and its environment. LPS is composed of three portions: lipid A, core oligosaccharide, and O-antigen. Whereas the lipid A moiety is the predominant cause of the endotoxic effects of LPS, the O-antigen is the most immunodominant portion of the molecule and thus has a profound effect on the interaction between a bacterial pathogen and the host organism. We have been characterizing the immune response to LPS by taking advantage of isogenic strains of *S. typhimurium* that differ only in a minor modification of O-antigen, namely acetylation. By examining the interaction of a series of monoclonal anti-LPS IgA antibodies, we have previously provided evidence suggesting that acetylation of O-antigen affects the three-dimensional structure of the molecule and thus creates and destroys a series of conformational antigenic determinants. Here we show that acetylation of O-antigen alters recognition by the vast majority of individual antibodies. This differential antibody recognition of O-antigen had a statistically significant correlation with protection against subsequent challenge with virulent *S. typhimurium*. In order to quantitate the antibody response against LPS, monoclonal antibodies were generated from mice that were immunized with whole bacteria. The majority of these monoclonal antibodies were directed against LPS and their recognition of LPS was also affected by the acetylation state of O-antigen. We are characterizing these monoclonal antibodies in detail. In particular, we are testing our hypothesis that acetylation of O-antigen creates a series of distinct epitopes. The development of this model system will allow us to address the induction of the immune response to this important non-protein antigen.

A SECRETED COLONIZATION FACTOR ASSOCIATED WITH THE *VIBRIO CHOLERAE* TOXIN COREGULATED PILUS

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Colonization of the human small intestine by *Vibrio cholerae* requires the type-4 Toxin Coregulated Pilus (TCP). Genes which encode for the structure and biogenesis functions of TCP are organized in an operon located within a large pathogenicity island termed the TCP-ACF element or Vibrio Pathogenicity Island (VPI). Tn*phoA* mutagenesis and DNA sequence analysis have defined the genes involved in TCP biogenesis, however the function of most of these gene products has yet to be well characterized and little is known about type-4 pilus biogenesis in general. In an effort to address these issues, in-frame deletions of genes within the *tcp* operon have been constructed and the resulting mutants characterized with respect to the assembly and function of TCP. Unique among these mutants are those which harbor an in-frame deletion of *tcpF*. Such mutants produce a normal amount of TCP that appear as morphologically wild-type, laterally associated bundles when examined in vitro, but have a severe colonization defect equivalent to that of a TCP-negative mutant in the infant mouse cholera model. Further examination reveals that TcpF is secreted into the culture supernatant during growth of *V. cholerae* under TCP-expressive conditions and that TcpF accumulates in the periplasm in *tcpA* or other mutants defective for TCP assembly, suggesting that its secretion outside the cell is dependent on TCP assembly. However, TcpF cannot mediate colonization independently of TCP, since a *tcpA* missense mutant that retains the ability to secrete TcpF but fails to elaborate a functional TCP remains defective for colonization. Attachment of a Δ*tcpF* mutant to cultured HT-29 differentiated human colonic epithelial cells occurs in a manner similar to wild-type, yet microcolonies formed on cell monolayers and during in vitro autoagglutination assays are slightly reduced in size indicating that TcpF may have a role in bacterial interactions. Based on these observations we conclude that TcpF is a secreted colonization factor localized extracellularly via a unique pathway which requires TCP assembly and that it contributes in a novel fashion to the colonization process of *V. cholerae.*

TRANSCRIPTIONAL ACTIVATION OF THE *VIBRIO CHOLERAE* TOXR VIRULENCE CASCADE REQUIRES COOPERATION BETWEEN APHA AND A LYSR HOMOLOG, APHB, AT THE *tcpPH* PROMOTER

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Transcriptional activation of the major virulence genes of *Vibrio cholerae* occurs via a regulatory cascade involving multiple factors encoded both within the "ancestral" chromosome and on discrete elements involved in pathogenicity. The toxin co-regulated pilus (TCP) genes are located on the TCP-ACF element, or VPI, and the cholera toxin (CT) genes are encoded on the lysogenic prophage CTXϕ. ToxR/ToxS are a chromosomally encoded activator pair which cooperate with the TCP-ACF encoded TcpP/TcpH activator pair to initiate the expression of another TCP-ACF encoded regulatory protein, ToxT. ToxT, in turn, directly activates expression of the *tcp* and *ctx* operons. We describe here a new chromosomally encoded protein pair, AphA/AphB, which functions early in the virulence cascade to activate the transcription of the *tcpPH* operon. AphA is a 20 kDa protein with no known homologs which functions synergistically in both *V. cholerae* and *E. coli* with AphB, a 33 kDa member of the LysR family of transcriptional regulators. Induction of *aphB*, but not *aphA*, from the *tac* promoter in El Tor biotype strains of *V. cholerae* dramatically increases the normally low levels of ToxR-regulated virulence gene expression observed in vitro to levels similar to those observed with classical biotype strains under all growth conditions examined. These results suggest that the ability of AphB to activate transcription from the *tcpPH* promoter in the presence of AphA is different in the two disease causing biotypes.

LOCALIZATION OF THE LAMININ-BINDING AND THE PROTEOLYTIC REGIONS IN THE PLASMINOGEN ACTIVATOR SURFACE PROTEASE PLA OF *YERSINIA PESTIS*

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Pla is a surface protease responsible for the highly invasive character of *Yersinia pestis*. Pla proteolytically activates plasminogen into plasmin and also inactivates the complement protein C3, both processes may affect the virulence potential of Pla. We recently found that Pla also is a laminin-specific adhesin, Pla does not degrade laminin but requires the presence of plasminogen to degrade laminin and basement membrane. This study was undertaken to dissect by genetic means the proteolytic and the adhesive functions as well as to localize the active amino acid residues within the Pla molecule.

Pla has 48% sequence identity with the *Escherichia coli* OmpT surface protease which has been proposed to function in processing of denatured proteins. Identification of transmembrane β-sheets suggested that Pla and OmpT have five surface accessible loop structures. *E. coli* XL1(pMRK2), expressing *ompT* from MG1655 did not activate plasminogen and adhered only poorly to laminin. In contrast, *E. coli* XL1(pMRK1) that expressed the *pla* gene on a 1.2 kb fragment from pPCP1 of *Y. pestis*, efficiently adhered to laminin and activated plasminogen. We located functionally active regions in Pla by constructing hybrid molecules where putative surface-associated loops were exchanged between Pla and OmpT. The other approach was to create amino acid substitutions at the sites in Pla suspected to be involved in proteolysis or adhesion. *E. coli* XL1 cells expressing the mutated or chimeric proteins were tested for plasminogen activation, proteolysis of a chromogenic substrate, adhesion to laminin, as well as surface expression of Pla/OmpT using immuno-fluorescence staining of XL1 derivatives and Western blotting of membrane proteins.

We obtained an amino acid substitution that was abolished in plasminogen activation but retained adherence to laminin, indicating that proteolysis and adherence can be dissected. Analysis of the Pla/OmpT hybrids showed that the N-terminal loops 4 and 5 in Pla are critical for laminin-binding, the plasminogen activation specificity is dependent on loops 3 and 4 of Pla. The catalytic triad involves amino acids in at least loops 2 and 4.

NADPH OXIDASE AND *Lgn1*^{R} ARE ESSENTIAL FOR EFFICIENT PREVENTION OF LETHAL *LEGIONELLA PNEUMOPHILA* PNEUMONIA

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Legionella pneumophila (*Lp*) is a causative agent of human pneumonia and can multiply in the phagosomes of monocytes/macrophages. In mice, *Lgn1* determines the extent of *Lp* growth in macrophages. The genotypes of C57BL/6 and A/J mice are *Lgn1*^{r} and *Lgn1*^{s}, respectively. On the other hand, oxidative burst is associated with *Lp* growth restriction in J774 macrophage-like cells (IAI 62: 5419, 1994). In the current study, we compared the role of two factors *in vivo*, Lgn1 and NADPH oxidase, using gp91*phox*$^{-/}$ mice. Male mice (*Lgn1*^{s}, gp91*phox*$^{-/}$) were obtained by backcrossing C57BL/6 gp91*phox*$^{-/+}$ female mice to A/J male mice more than 5 times. A LD_{50} value of mice in the intranasal infection was markedly low only in the mice of (gp91*phox*$^{-/}$, *Lgn1*^{s}) genotype, most of which could not clear *Lp* from the lungs for 8 days after infection. The mice of gp91*phox*$^{-/}$ but not gp91*phox*$^{+/}$ were observed to retain the *Lp* in the spleen and liver, indicating that NADPH oxidase plays an important role in killing *Lp* in the early stage of infection. These results suggest that oxidative burst and macrophage natural resistance against *Lp* are essential for efficient prevention of lethal *Lp* pneumonia and that our mice presented here are a useful model for Legionnaires' disease.

LD_{50}	*Lgn1*^{r}	*Lgn1*^{s}
gp91*phox*+	1.3×10^7 cfu	7.6×10^6 cfu
gp91*phox*-	2.2×10^6 cfu	4.5×10^4 cfu

USE OF TRANSPOSON FOOTPRINTING FOR IDENTIFICATION OF BACTERIAL VIRULENCE GENES

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Comprehensive screening of the bacterial genome for virulence genes has not been a feasible goal using standard genetic methods. Signature-tagged mutagenesis has been proven very useful for this application but still is limited by the pool sizes that can be screened. In an effort to devise a more comprehensive method to screen bacterial genomes for virulence genes, we developed a transposon footprinting method that simultaneously amplifies each of the transposon-flanking sequences present in a complex pool of transposon mutants. Because the length of the amplified DNA should be unique for each distinct transposon insertion, each DNA fragment separated on a gel represents the corresponding transposon insertion. We used this method to generate transposon footprints of Tn5 mutant pools of *Salmonella typhimurium* 14028s. Tn5 mutants of *S. typhimurium* which are missing from the original pool (n=100) could be detected easily by comparing the transposon footprints of the two pools. Currently, we are combining this transposon footprinting method with PCR-based subtractive hybridization to efficiently detect the differences in transposon insertions in two complex pools of transposon mutants. This method will be optimized for screening the genome of *S. typhimurium* for the virulence genes required for *in vivo* survival during mouse infection.

REGULATION OF SHORT-CHAIN FATTY ACID-INDUCED ACID RESISTANCE OF *SALMONELLA TYPHIMURIUM* BY REDUCTION-OXIDATION POTENTIAL

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Acid resistance is an important virulence phenotype required for the successful pathogenesis of *Salmonella typhimurium*. We previously reported that acid resistance of *S. typhimurium* is greatly induced by exposure to short-chain fatty acids (SCFA). One of the characteristic conditions in the gastrointestinal tract, where the exposure of *S. typhimurium* to SCFA is most likely to occur during its life cycle, is negative reduction-oxidation (redox) potential. In this study, we found that the negative redox potential further enhances the SCFA-induced acid resistance of *S. typhimurium*. Acid resistance of *S. typhimurium* 14028s was determined after overnight culture grown in aerobic TSB was exposed to propionic acid (100 mM) for 1 hr in anaerobic TSB (pH 7.0) reduced to different levels by addition of reducing agents. The acid resistance of *S. typhimurium* as measured by % survival after 1 hr in TSB (pH 3.0) was 91±16, 164±18, 275±176 and 220±112 when exposed to propionic acid in aerobic TSB (redox potential: 66±9), anaerobic TSB with no reductant (23±16), cysteine (-43±14) or ascorbic acid (-73±10), respectively. The results indicate that the negative redox potential in the gastrointestinal tract of animal may enhance the virulence of *S. typhimurium* by increasing SCFA-induced acid resistance.

THE *Rhizobium meliloti* BacA PROTEIN AND HOMOLOGS: INVOLVEMENT IN HOST-MICROBE INTERACTIONS IN SYMBIOSIS AND PATHOGENESIS

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The *Rhizobium meliloti bacA* gene, which encodes a predicted membrane transporter, is required for nodule development in nitrogen-fixing *R. meliloti*-alfalfa symbioses. *BacA* mutants senesce at an intermediate time-point in symbiotic development, when wild type bacteria would normally begin to differentiate into nitrogen-fixing bacteroids. We hypothesize that BacA is involved in critical signal exchange between endosymbiont and host. BacA is closely related to, and isofunctional with, the *E. coli sbmA* gene, which encodes a protein implicated in the transport of some antibiotics.

In order to explore the potential significance of BacA homologs in other host-bacterial relationships, we searched for and identified BacA homologs from the mammalian pathogens *Brucella abortus* and *Salmonella typhimurium*. *B. abortus bacA* mutants had decreased survival levels in murine macrophages *in vitro*, and greatly attenuated pathogenicity in mice, with mutants persisting at levels 4 to 5 orders of magnitude lower than wild type at eight weeks post infection, suggesting that BacA is necessary for chronic *B. abortus* infection in mice. We are in the process of carrying out pathogenicity and invasion studies with *S. typhimurium bacA*(*sbmA*) mutants.

As *R. meliloti* BacA is required for symbiosis and *B. abortus* BacA is necessary for chronic infections in murine phagocytes, these proteins may be involved in alerting bacteria to their host environments, prompting cellular changes that allow survival within eukaryotic cells.

O- ANTIGEN SWITCHING IN *VIBRIO CHOLERAE* BY HOMOLOGOUS RECOMBINATION

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The DNA sequence of the O-antigen biosynthesis cluster (*wbf*) of the recently emergent pathogen, *Vibrio cholerae* serogroup O139, has been determined. The 35 kb *wbf* gene cluster (28 ORFs) begins at *wbfA,* immediately after *gmhD* and ends at *wbfX*. The gene downstream of *wbfX*, designated *rjg* (*right junction gene*), is predicted to be not required for O-antigen biosynthesis but appears to be a hot spot for DNA rearrangements. Several variants of the *rjg* gene (three different IS insertions and a deletion) have been found in other serogroups. DNA dot blot analysis of 106 *V. cholerae* strains showed the presence of the left and right junction genes *gmhD* and *rjg*, respectively, in all the strains. Further, these genes mapped to a single I-*Ceu*I fragment in all the 21 strains analyzed by PFGE, indicating a close linkage. The IS element, IS*1358,* found in O1 and O139 *wb** regions, is present in 61% of the strains tested and interestingly, where present, it is predominantly linked to the *wb** region. These results indicated a cassette-like organization of the *wb** region, with the conserved genes, *gmhD* and *rjg,* flanking the divergent, serogroup-specific *wb** genes and IS*1358*. Similar organization of the *wb** region in other serogroups raises the possibility of the emergence of new pathogens by homologous recombination via the junction genes. Results of testing this prediction by switching the O-serogroup of a non-O1/ non-O139 strain to O139 will be discussed.

AN IMPORTANT ROLE FOR THE CorA MAGNESIUM TRANSPORTER IN BACTERIAL VIRULENCE

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CorA is the primary magnesium uptake protein in Eubacteria and Archaea. A number of loci in *Salmonella typhimurium* which are known to be induced upon invasion of eukaryotic cells, including the Mg^{2+} transport loci *mgtA* and *mgtCB*, are regulated by the PhoPQ two component sensor-kinase, known to be important for virulence, as are *mgtB* and *mgtC*. The presence or absence of a functional *corA* allele also modulated the expression of *mgtA* , *mgtCB*, and other phoPQ-regulated genes in free-living bacteria. Expression of these same loci was also modulated by the presence of a functional *corA* allele upon *S. typhimurium* infection of J774 macrophage-like cell line. Further, in *Escherichia coli*, *corA* appears to be a heat shock gene (F. Blattner, personal communication). We determined that in a strain carrying a *corA* deletion, *S. typhimurium* was markedly tolerant to heat shock relative to wild type, being able to survive 20 min exposure to 55 °C with good viability. These data are tentatively interpreted to suggest that maintenance of high intracellular Mg^{2+} levels are important for viability, heat resistance and virulence of bacteria. Therefore, the CorA magnesium transporter may be a good target for antimicrobials.

USE OF GREEN FLUORESCENT PROTEIN (GFP) TO STUDY THE INVASION PATHWAYS OF *EDWARDSIELLA TARDA* IN *IN VIVO* AND *IN VITRO* FISH MODELS

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Edwardsiella tarda is a fish pathogen that causes systemic infections in many food and ornamental fishes. *E. tarda* PPD130/91 and PPD125/87 were selected as representatives of the virulent and avirulent groups respectively from eight fish isolates, and transformed with plasmids encoding either green fluorescent protein (pGFPuv) or blue fluorescent protein (pBFP2). Two host models were used to study the invasion pathway of *E. tarda in vitro* and *in vivo*. Epithelioma papillosum of carp (EPC) was used as the first model. Virulent and avirulent *E. tarda* strains were found to adhere to and invade EPC cells. Both, interactions between *E. tarda* and host cells examined under confocal microscopy, and intracellular growth were followed at different time points. Bacterial internalisation of PPD130/91 and PPD125/87 involved microfilament and protein tyrosine kinase as cytochalasin D (inhibitor of microfilament polymerisation) and genistein (inhibitor of protein tyrosine kinase) prevented internalisation. Confocal studies revealed co-localisation of polymerised actin with bacteria. Staurosporine, a protein kinase C inhibitor, accelerated internalisation of PPD125/87, whereas PD098059, a MAPK kinase inhibitor prevented internalisation of PPD130/91. In the second model, blue gourami were infected with *E. tarda* intramuscularly. Mortalities were observed in PPD130/91(pGFPuv)-infected fish with high bacterial numbers detectable in all organs. PPD125/87(pBFP2)-infected fish did not die and the bacterial population dropped over time. Mixed infections where inoculum size was similar to the single infections, comprised of both PPD130/91(pGFPuv) and PPD125/87(pBFP2) caused mortalities in fish. High bacterial populations were noted only in the fish body muscle. PPD125/87(pBFP2) population in the fish dropped eventually. The number of PPD130/91(pGFPuv) also dropped in the fish organs, except for continued high growth in the body muscle. Histology revealed necrosis of the tissue (body muscle and liver) and fluorescent bacteria in fish that were infected with PPD130/91(pGFPuv) but not PPD125/87(pBFP2). This study showed that fluorescent proteins are a good tool for investigating bacterial-host cell infection and information elucidated here sheds new light on the interactions between *E. tarda* and their hosts.

THE TIR-BINDING REGION OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* INTIMIN IS SUFFICIENT TO TRIGGER ACTIN CONDENSATION AFTER BACTERIAL-INDUCED HOST CELL SIGNALING

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Enterohemorrhagic *E. coli* (EHEC) has emerged as an important agent of diarrheal disease in the U.S. Epithelial cell attachment by EHEC, as well as by several other enteric pathogens such as enteropathogenic *E. coli* (EPEC), is associated with the actin condensation and the formation of "attaching and effacing" (A/E) lesions on the surface of epithelial cells (reviewed in [1]). The bacterial outer membrane protein intimin, encoded by *eae*, is a bacterial ligand that is critical for the formation of this structure.

A receptor for intimin, termed Tir, is a bacterial protein that is translocated to the host cell surface [2]. However, intimin is homologous to the *Yersinia pseudotuberculosis* invasin, which binds several β_1-chain integrins, and the finding that recombinant intimin also binds to β_1-integrins raised the possibility that both integrins and Tir could function as intimin receptors [3]. We found that mammalian cells did not bind to immobilized EHEC intimin unless they were preinfected with EPEC or EHEC. β_1-chain integrin antagonists or inactivation of gene encoding the β_1-chain did not affect intimin-mediated cell binding or actin condensation [4].

To better understand Tir-intimin binding, we assayed this interaction in a yeast two-hybrid system. Deletion analysis indicated that the central region of Tir that is flanked by two putative transmembrane segments was sufficient to promote transcriptional activation in the two-hybrid system. This activation reflected a direct interaction between the proteins, because a recombinant protein carrying a 65-residue segment of Tir from the central region bound to recombinant intimin in vitro.

Finally, to better understand the requirements of intimin to trigger cell binding and A/E lesion formation, we identified the cell binding and outer membrane localization domains of intimin [5]. The N-terminal 539 amino acids of intimin were sufficient to promote outer membrane localization of the C-terminus of *Y. pseudotuberculosis* invasin. The C-terminal 181 residues of intimin were sufficient to bind mammalian cells that had been preinfected with EPEC *eae*$^-$. Binding of intimin derivatives to preinfected cells correlated with binding to recombinant Tir protein, and the 181-residue minimal Tir-binding region of intimin, when purified and immobilized on latex beads, was sufficient to trigger A/E lesion formation on preinfected mammalian cells.

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HILA-DEPENDENT ACTIVATION OF THE INVF PROMOTER

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HilA, a transcription factor encoded on *Salmonella* Pathogenicity Island 1 (SPI-1), is required for the expression of invasion genes. Genetic evidence suggests that a subset of these promoters is activated directly by HilA. Activation of the (SPI-1 encoded) *prgH* and *invF* promoters by HilA can be reconstituted in *E. coli* expressing HilA from a plasmid. Truncation and mutation studies of both of these promoters in *E. coli* suggests that a symmetrical sequence immediately upstream of the –35 regions of these promoters is a HilA binding site (HilA Box). *InvF* promoters with mutations in or deletions of the HilA Box no longer respond to HilA. Extracts made from *E. coli* expressing HilA under the control of an arabinose promoter change the apparent electrophoretic mobility of *invF* promoter DNA. Competition experiments demonstrate that the HilA Box is necessary and sufficient for HilA to shift the *invF* promoter DNA in this assay. These results are strong genetic and biochemical evidence that HilA is a transcription factor that interacts directly with specific sequences in the invF promoter.

POTENTIAL REGULATORS OF INVASION GENE EXPRESSION IN *SALMONELLA TYPHIMURIUM*

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An important early step in *Salmonella* pathogenesis is the invasion of intestinal epithelia. Upon contact with host cells, *S. typhimurium* produces proteins and injects them directly into host cells by a Type III secretion system, inducing morphological changes in the host cell and uptake of the bacteria by normally non-phagocytic epithelial cells. Many of the genes encoding the secretion apparatus and the secreted proteins are located on a 40 kb pathogenicity island (SPI1) at minute 63 of the *S. typhimurium* chromosome. Coordinate regulation of these invasion genes by a variety of environmental conditions and regulatory mutations is thought to be mediated by altering expression of a transcriptional regulator, *hilA*. To identify potential upstream sensors and regulators of *hilA* and invasion genes, mutations were isolated that cause decreased *hilA* and/or *sipA* expression. One such mutation, *pstS::Tn5*, is complemented for its effects on *hilA* and invasion gene expression by adding a plasmid containing the *E. coli pst-phoU* operon. The Pst phosphate uptake system is required for repression of the transcriptional regulator PhoB, suggesting that invasion genes are repressed by PhoB. This is supported by the finding that a mutation in *phoB* suppresses *pstS::Tn5*'s effect on *hilA* and invasion gene expression. Another Tn5 insertion was isolated in *fadD*, the gene encoding acyl coenzyme A synthetase. Addition of a plasmid expressing the *E. coli fadD* restores normal *hilA* and invasion gene expression to this mutant. Acyl Co-A synthetase is required for transport of long chain fatty acids (LCFAs) into the cytoplasm and for metabolizing LCFAs via B-oxidation. Acyl-CoA can prevent the transcriptional regulator FadR from binding to promoters, but the decreased expression of *hilA* and invasion genes in the *fadD* mutant does not seem to be due to repression by FadR since a mutation in *fadR* does not suppress the effects of the *fadD* mutation on invasion gene expression. Two Tn5 insertions in *flhC*, one in *flhD*, and two in *fliA* have also been identified. The decreased *hilA* expression in these mutants is alleviated by adding a plasmid expressing *E.coli fliZ*, a gene of unknown function located downstream of *fliA*.

IN VITRO AND IN VIVO CHARACTERIZATION OF HETEROLOGOUS *eaeA* ALLELES IN AN *eaeA* MUTANT STRAIN OF THE MOUSE PATHOGEN *CITROBACTER RODENTIUM*

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Differences exist among attaching and effacing (A/E) species in terms of host specificity, intestinal tissue tropism, and disease pathogenesis. The divergence seen in the C-terminal region of the intimin protein has been postulated to account for some of these differences. Indeed, previous work has specifically investigated the impact of intimin alleles on disease pathogenesis. Tzipori and coworkers trans-complemented an EHEC O157:H7 *eaeA* mutant with a plasmid-encoded intimin allele from an EPEC strain. In the gnotobiotic piglet, this strain caused an EPEC-like colonization pattern and disease, suggesting that intimin allelic differences could play a role in defining disease pathogenesis. Frankel and coworkers extended this work by testing the same plasmid-encoded EPEC intimin allele in a *C. rodentium eaeA* mutant. A mouse infection study showed reduced virulence compared to the wild-type *C. rodentium* strain and a colonization pattern that differed only in the crypt depth at which the trans-complemented strain adhered. A limitation to both of these studies was the lack of controls for the effects of gene copy number and regulation of gene expression from a plasmid-encoded allele.

Our objective is to investigate the impact that differing alleles of the intimin-invasin superfamily of proteins have on A/E lesion formation, intestinal tissue tropism, and disease pathogenesis in the *C. rodentium* mouse model of A/E disease. In order to accomplish this, we have generated a series of plamids that control for the difficulties caused by plasmid copy number and regulation of gene expression from a plasmid-encoded allele. These constructs have been characterized *in vitro* for expression of the intimin protein and for the ability to focus actin beneath adherent bacteria. Further characterization has been performed *in vivo* to assess the site and extent of colonization and the hyperplastic response in the mouse.

TRANSCRIPTIONAL REGULATION OF THE *MANNHEIMIA (PASTEURELLA) HAEMOLYTICA* LEUKOTOXIN

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Mannheimia (Pasteurella) haemolytica is a commensal bacterium of the upper respiratory tract of healthy cattle. However, when the animals are stressed, the bacteria can cause a severe respiratory pneumonia by rapidly multiplying and descending to the lungs. Leukotoxin is one of the primary virulence factors of *M. haemolytica,* and it is proposed to have increased expression in the lung. We are studying the transcriptional control of leukotoxin expression to identify factors and conditions associated with *M. haemolytica's* conversion to virulence.

The leukotoxin operon is preceded by a 405 bp promoter region that is shared by the divergently transcribed arginine binding protein gene (*lapT*). Several DNA elements within the promoter region may be involved in transcription control, including a site for binding of a putative activator, a static DNA bend, and a consensus binding site for the DNA-bending protein, integration host-factor (IHF). To study *cis*- and *trans*-acting factors involved in leukotoxin transcription we have created a genetic system for transcriptional studies in *M. haemolytica*. Leukotoxin chloramphenicol acetyl transferase (*cat*) operon fusions and a CAT assay have been developed. Using this system we have shown that IHF is involved in regulation of *lapT* transcription while it has no affect on leukotoxin expression in *M. haemolytica*. We have found, however, that H-NS, a member of the histone-like protein family, which binds to DNA bends, represses leukotoxin transcription in *E. coli*. Nested deletions are being created to locate binding sites for IHF and H-NS and to identify other important DNA transcriptional elements. To identify activators of leukotoxin transcription, a *trans*-complementation assay was developed and a *M. haemolytica* library was screened in *E. coli*. Several clones that could contain a leukotoxin activator have been isolated and are being characterized. This study will identify factors important for the regulation of leukotoxin transcription and should lead to a better understanding of *M. haemolytica's* switch from a commensal to a pathogenic organism.

CRYSTAL STRUCTURE OF*LISTERIA MONOCYTOGENES* INTERNALIN-B LRR DOMAIN, A BACTERIAL PROTEIN INVOLVED IN MAMMALIAN CELL INVASION.

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Listeria monocytogenes, a facultative intracellular bacterial pathogen, invades several types of mammalian cells that are normally not phagocytic by a mechanism termed 'induced phagocytosis'. Invasion of hepatocytes, endothelial, and certain epithelial cell types is mediated by the interaction of a *Listeria* cell wall-attached protein, internalin B, with a receptor on the mammalian cell surface. This interaction causes activation of the host cell phosphoinositide (PI) 3-kinase pathway and subsequent changes in the host actin cytoskeleton, culminating in phagocytosis of the pathogen. Internalin B belongs to the large family of *Listeria*-encoded internalin proteins, many of which, including internalin B, are required for virulence.

Internalin B contains a leucine-rich-repeat (LRR) domain that is necessary and sufficient to trigger phagocytosis and activate PI 3-kinase. We have determined the 1.86 Å resolution x-ray crystallographic structure of this mammalian cell effector domain to elucidate the structural features responsible for inducing phagocytosis. The structure reveals that the domain is elongated and curved, containing a concave surface that has a likely role in interacting with its mammalian receptor. In addition, calcium ions are observed bound to the domain in a manner suggestive of a role in protein-protein interactions. The structure of the LRR domain provides a framework for understanding the internalin family of virulence factors at the molecular level.

GENETIC ANALYSIS OF *E. COLI* K1 GASTROINTESTINAL COLONISATION.

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Escherichia coli K1 is the leading cause of Gram-negative neonatal meningitis. The bacterium is acquired by infants from their mothers or from health-care workers. Using serotype analysis, isolates of *E. coli* K1 recovered from the cerebrospinal fluid of affected infants can be identified simultaneously in the gastrointestinal (GI) tract of mothers and infants. Some of the bacterial factors that are required for systemic disease have been defined, however the mechanisms of acquisition and colonisation of *E. coli* K1 have been largely unstudied. We have used signature-tagged mutagenesis to identify genes that are involved in GI colonisation.

A library of 2,220 insertional mutants was constructed in *E. coli* K1 using mTn*5* harbouring pre-selected signature tags. The mutants were screened for their ability to establish GI colonisation in the infant rat model. Five day-old animals were inoculated in duplicate intra-gastrically with 5×10^8 cfu consisting of 48-96 mutants. The profile of mutants present in the inoculum was compared with the mutants recovered from the large bowel of animals 48 hr after inoculation. We identified a total of 15 mutants that are defective in GI colonisation. The insertion site of the transposon in these mutants was characterised, and revealed insertions in genes encoding cell surface structures (2), regulators (3), enzymes involved in metabolic pathways (5), and genes of unknown function (5). We have further characterised the nature of the colonisation defect in the mutants by examining their fate in the GI tract after inoculation using immunohistochemistry. One mutant, affected in LPS biosynthesis, appears in the small bowel within an hour of inoculation but fails to persist. A further mutant, carrying an insertion in a gene of unknown function, can be detected in both the small and large bowel throughout experiments but in much reduced numbers compared to the wild-type strain.

NEISSERIAL PORIN INTERACTS WITH MITOCHONDRIA AND PROTECTS CELLS FROM APOPTOSIS

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The major outer membrane protein of the pathogenic Neisseria, termed porins or Por, can act as B cell mitogens and an immune adjuvant. The mechanism of their stimulatory ability is unknown. To explore the possible mechanisms of their stimulatory activity and to begin to discern the specific interactions of Por with eukaryotic cells, the fate of meningococcal PorB, after its interaction with lymphocytes was examined . Murine B cells (CH12-RMC), human Jurkat cells and murine splenic B cells were incubated in medium or with purified PorB for 24h; membrane and cytosolic fractions were obtained and analyzed by SDS-PAGE. Immunoblotting with anti-PorB polyclonal serum demonstrated the presence of PorB only in the membrane fractions. Sub-membrane fractionation interestingly showed PorB association with isolated mitochondria. Mitochondrial membrane depolarization was induced by treatment with the apoptotic inducing substance, staurosporine, and the effect of PorB on mitochondrial membrane potential ($\Delta\Psi_m$) was examined. Preincubation of CH12 RMC or Jurkat cells with PorB for 24h before staurosporine treatment, reduced the loss of $\Delta\Psi_m$. CH-12 RMC cells showed mitochondrial volume loss during staurosporine treatment, which was prevented by PorB preincubation. Staurosporine-mediated loss of $\Delta\Psi_m$ and release of cytochrome c can lead to activation of caspases involved with apoptosis and DNA laddering is one of the end results of this process. PorB pre-incubation of Jurkat and CH12-RMC cells reduced the release of cytochrome c into the cytosol induced by treatment with staurosporine. In addition, PorB pre-incubation of cells prior to treatment with staurosporine partially prevented DNA laddering. In this work we show that neisserial porin PorB has a stabilizing effect on mitochondria during apoptosis. Induction of target cell apoptosis constitutes an essential part of antigen-specific immune effector mechanism and inhibition of programmed cell death of pathogen stimulated immune cells could be advantageous to the host. This finding could be related to the neisserial porins' effects on B cell function including proliferation, antibody secretion and may help discern the mechanism of their ability to activate antigen presenting cells and induce cellular modifications.

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SPONTANEOUS TANDEM AMPLIFICATION AND DELETION OF THE SHIGA TOXIN OPERON IN SHIGELLA DYSENTERIAE 1

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Among the species and serotypes of *Shigellae*, only *S. dysenteriae* 1 contains the genes encoding Shiga toxin (Stx). Closely related toxins are produced by Shiga toxin-producing *Escherichia coli* (STEC). In STEC, the *stx* genes are usually carried by lambdoid bacteriophages, and are highly mobile. In contrast, *stx* in *S. dysenteriae* 1 has been believed to be chromosomally encoded and not transmissible. We have re-examined the mobility of *stx* and the relationship between the STEC and *S. dysenteriae* 1.

Analysis of a 32.1 kbp chromosomal fragment from *S. dysenteriae* 1 strain 3818T located the toxin genes within a region homologous to minute 30 of the *E. coli* chromosome. Lambdoid bacteriophage genes with homology to regions of the STEC bacteriophages H19B and 933W surround the *stx* operon. These genes are present in all *S. dysenteriae* 1 strains examined, and are absent from other *Shigella* species. Three pairs of identical IS elements flank the *stx* genes, interrupting the bacteriophage genes and placing the toxin operon within a 22.6 kbp putative composite transposon bracketed by identical IS600 insertion sequences.

We provide the first demonstration that the *stx* operon can exist in multiple copies on the *S. dysenteriae* 1 chromosome. Tandem amplification of up to ten copies of the *stx* region occurs via recombination between the flanking IS600 insertion sequences, leading to increased toxin production. Amplification is easily selected in a strain with an antibiotic-marked *stxAB*, but also occurs spontaneously in unmarked laboratory and newly isolated clinical strains. Toxin gene copy number under different growth conditions has been assessed using quantitative PCR. A linkage between chlorate-resistant mutants of *S. dysenteriae* 1 and the loss of Stx production has been known for many years but not understood at the molecular level. We have explained this linkage by demonstrating that the global regulatory gene *fnr* lies within the *stx* region. Therefore, deletions of the toxin genes, which occur by recombination between the flanking IS600 elements, are selected by anaerobic growth on chlorate-containing medium.

Using an antibiotic-marked H19B phage, we have demonstrated that STEC bacteriophage easily lysogenize *Shigella* species. We propose that *S. dysenteriae* 1 was originally lysogenized by an Stx-encoding bacteriophage, which became defective due to loss of sequences following IS element insertions and rearrangements. These insertion sequences have subsequently allowed the amplification and deletion of the *stx* region.

ROLE OF MAST CELLS IN EARLY IMMUNE RESPONSE TO *BORDETELLA PERTUSSIS* INFECTION

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Purpose: Since recent studies have demonstrated the role of mast cells in the host defense against bacterial infections, particularly in the lungs, we wanted to determine whether *Bordetella pertussis,* the pathogen responsible for whooping cough, could interact with mast cells and investigate the influence of this interaction on the early immune response during infection.

Results: Following exposure to wild-type or pertussis toxin (PT)-deficient *B. pertussis,* bone-marrow derived mast cells (BMMC) released proinflammatory cytokines such as TNFα and IL-6. In addition, the PT-deficient strain, but not the wild type strain, induced the release of the Th2 cytokines IL-4 and IL-10. By using electron microscopy we found that both bacterial strains were internalized by BMMC and killed during the first hour following contact. Nevertheless, some bacteria were able to escape from the phagocytic vacuole and were found free in the cytoplasm of BMMC. Moreover and in agreement with recent studies demonstrating that mast cells express MHC class II and costimulatory molecules, we found that infected BMMC are able to present *B. pertussis* antigens to T cells purified from *B. pertussis*-infected lungs.

Conclusion: In this study, we have shown that the intensity and the type of cytokines released by BMMC following exposure to *B. pertussis* is influenced by PT expression by the bacteria. Considering the fact that BMMC share many similarities with mucosal mast cells, we propose that mucosal mast cells contribute to the early immune responses during respiratory infection with *B. pertussis.* We have also shown that BMMC can internalize and kill *B. pertussis.* Nevertheless, some bacteria were found free in the cytoplasm suggesting that mast cells could also be a reservoir for *B. pertussis.*

QUORUM SENSING IN *VIBRIO CHOLERAE*

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In order to adapt to a fluctuating environment, bacteria have the ability to detect multiple environmental inputs and to integrate this information to effect an appropriate response. The model bacterium, *V. harveyi*, modulates the expression of bioluminescence (*lux*) in response to a variety of environmental cues, one of which is cell density. Regulation of gene expression in response to cell density is a phenomenon known as quorum sensing. Quorum sensing is controlled by the secretion of and response to extracellular signaling molecules called autoinducers. In *V. harveyi*, two different autoinducers have been identified, and they transduce population density information to the cell through parallel two-component systems. The autoinducer signals converge at a shared integrator protein that controls *lux* expression. Our results suggest that autoinducer 1 (AI-1) regulates intra-species cell-cell signaling, whereas autoinducer 2 (AI-2) is responsible for inter-species cell-cell communication. We have demonstrated that many bacteria produce an AI-2 activity that can stimulate luminescence expression in *V. harveyi*. Furthermore, we have identified and cloned the gene responsible for AI-2 production (*luxS*) in many genera of bacteria. These bacteria include *V. harveyi*, *V. cholerae*, *Escherichia coli* and *Salmonella typhimurium*. We propose that AI-2 is involved in regulating quorum sensing in all of the identified species of bacteria. One focus of our research is to determine the role of quorum sensing in *V. cholerae*. Our data suggest that AI-2 is involved in the regulation of pathogenicity in *V. cholerae*, because both classical and El Tor *V. cholerae luxS* null mutants are attenuated in virulence in mice. We are in the process of identifying the genes that are regulated by AI-2 in *V. cholerae*, identifying the *V. cholerae* quorum sensing signal transduction circuit, and establishing the environmental conditions that modulate AI-2 production in *V. cholerae*. The molecular characterization of quorum sensing in *V. cholerae*, along with a comparison to the *V. harveyi* quorum sensing system, could reveal novel mechanisms that distinguish free-living non-pathogenic bacteria from pathogens.

TRICLOSAN INHIBITION OF MYCOBACTERIA SUGGESTS THAT SYNTHESIS OF BOTH MYCOLIC ACIDS AND FATTY ACIDS ARE TIGHTLY LINKED .Héctor R.Morbidoni, Jeffery S. Cox and William R. Jacobs, Jr. Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York.

Enoyl-ACP Reductases are emerging as an important target for the design of new antimicrobial drugs. These proteins performs an essential role in the synthesis of fatty acids. Mycobacteria are unique since they use two different systems for the synthesis of fatty acids, one involved in the synthesis of medium chain length fatty acids (up to C26 in length),designated FAS I, and the other one responsible for the synthesis of very long α-alkyl β-hydroxy fatty acids mycolic acids, designated FAS II. It has previously been shown that the synthesis of mycolic acids is inhibited by the anti-tuberculous drugs ethionamide and isoniazid. The target for both drugs is InhA, an enoyl-ACP Reductase with specificity for long chain fatty acids. Mutations in *inh*A are sufficient to confer resistance to isoniazid and ethionamide. Triclosan, a tri-chlorinated diphenyl ether has been recently shown to act as an enoyl-ACP Reductase inhibitor in *E.coli* and in *M.smegmatis*, a fast-growing saprophytic mycobacteria. This compound shows a remarkable similarity to isoniazid action on mycobacteria, except that it was reported as inactive against *M.tuberculosis* which displays an exquisite sensitivity to isoniazid. Here we show that triclosan is active on *M.avium*, *M.tuberculosis* and *M.tuberculosis* var. BCG. The Minimum Inhibitory Concentration (MIC) values for all the species tested were comparable to the MIC for *M.smegmatis*, in the range of 6 to 10 μg/ml. While a *katG*-encoded catalase-peroxidase is necessary for the conversion of isoniazid to the active form, triclosan does not require activation. Consistant with that hypothesis, triclosan is shown to be active on *katG* mutants. Since this class of mutants arise at very high frequency upon treatment with isoniazid, triclosan constitutes a clinically important alternative. Fatty acid analysis revealed that comparable concentrations of triclosan simultaneously inhibited both fatty acids and mycolic acids biosynthesis in all the species tested. The same result was also found upon incubation of a *M.smegmatis* *inh*A^{ts} mutant at non-permissive temperature. Mutations in *inh*A are sufficient to confer resistance to triclosan in *M.smegmatis* thus a direct inhibition of the enoyl-ACP Reductase domain of FAS I by triclosan seems unlikely. Taken together these results indicate that a block in the FAS II systems creates the conditions needed to shut down the synthesis of fatty acids. This metabolic link between both synthetases might offer new possibilities for the design of novel therapeutic agents.

SPI1 HAS FUNTIONS ALTERNATIVE TO INVASION DURING INFECTION

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Salmonella enterica sv. Typhimurium (*S. typhimurium*) causes typhoid-like symptoms in mice. Following ingestion, bacteria colonize the intestinal tract, penetrate the intestinal epithelium and access systemic sites such as the spleen and liver through the lymphatic and blood circulation. Traversion of the intestinal wall is believed to be initiated by bacterial invasion into enterocytes and M cells in the Peyer's patches. Invasion of cultured epithelial cells has been used as a model for invasion during *Salmonella* infection. This model has revealed that the invasion of non-phagocytic cells is mediated by a type III secretion system encoded by invasion genes on a 40-kb pathogenicity island at minute 63 of the *S. typhimurium* chromosome, *Salmonella* Pathogenicity Island 1 (SPI1).

We have studied the role of invasion genes during infection. The recovery of CFUs from infected tissues was compared between invasion mutant and WT strains. We used a strain with a deletion of SPI1 and a strain with a mutation on *hilA*, a transcriptional activator of several invasion genes. Single and mixed infections using these different strains followed by harvesting of tissues at different times have revealed interesting aspects of *Salmonella* pathogenesis. The *hilA* mutant strain was recovered at low frequencies at early times post-inoculation as compared to WT. The deletion strain was recovered at high frequencies at all times post-inoculation as compared to WT. Intragastric virulence, on the other hand, was reduced for the deletion strain but was unchanged for the *hilA* mutant. The infection phenotypes of the WT, *hilA*, and *ΔspiI* mutant strains can be explained by speculating that two important events occur when *S. typhimurium* infects its host. One, the host responds to the presence of *S. typhimurium* and attempts to clear the infection. Two, the bacteria express *hilA* and invasion genes which help them overcome the host clearance response. Our results suggest that the host might detect *S. typhimurium* by its expression of a SPI1 gene (or genes) that is present in both the WT and *hilA* mutant strains but that is deleted in the *ΔspiI* strain.We propose that the *ΔspiI* strain expresses no SPI1 factors and even in the absence of *hilA* can infect the host possibly through a different route of invasion. In addition it avoids triggering host clearance mechanisms. Our work emphasizes the importance of using several *in vivo* approaches and bacterial mutants to study the role of bacterial genes in pathogenesis.

PATHOGENICITY, VIRULENCE AND AVIRULENCE IN THE RICE BLAST FUNGUS *MAGNAPORTHE GRISEA.*

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The ascomycete *Magnaporthe grisea* causes disease on a wide variety of graminaceous hosts, although individual strains of the fungus are limited to infecting one or two grass species. Some examples of host-pathogen specificity in this system appear to fit the classical gene-for-gene hypothesis. The avirulence gene *AVR-Pita* (formerly *AVR2-YAMO)* confers avirulence towards the rice cultivar Yashiro-mochi, which contains resistance gene *Pi-ta*. Pathogen strains containing *AVR-Pita* fail to infect Yashiro-mochi, presumably due to the production of a signal molecule that interacts with *Pi-ta* and mediates recognition. *AVR-Pita* is predicted to encode a secreted processed zinc metalloprotease, mutation of specific amino acid residues in the putative zinc-binding region removes the ability to confer avirulence. Sequence comparisons with virulent *avr-pita* alleles from rice pathogens also indicate that maintenance of the putative zinc protease motif is essential. Rice plants treated with recombinant AVR-Pita protein showed no response suggesting that the *AVR-Pita* mediated signal may be on the inside of the plant cell. The rice *Pi-ta* resistance gene encodes a predicted cytoplasmic 928 amino acid protein containing a nucleotide binding site and a leucine rich N-terminus. Two hybrid and far western analysis demonstrate that there is a physical interaction between the AVR-Pita and Pi-ta proteins in vitro. Using a rice seedling particle bombardment transient assay we have demonstrated that AVR-Pita is specifically recognized within the plant cell by the *Pi-ta* gene product leading to recognition and initiation of the host defense response.

BACTERIAL CROSSTALK: INTERACTION BETWEEN YERSINIA AND ITS TARGET CELL

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Many pathogenic bacteria secrete various virulence associated proteins during the infectious process. Different Gram- bacteria including both animal and plant pathogens secrete effector proteins, directly affecting the host, via a common contact induced Type III secretion system. We are using *Yersinia pseudotuberculosis* as a model to understand the basic mechanisms behind the cell-contact dependent induction and subsequent polarized translocation of the effector proteins (Yops) into the cytosol of the target cell. *Y. pseudotuberculosis* up-regulates Yop-expression after intimate contact between the pathogen and its target cell has been established. This crosstalk involves also the Type III secretion machine. Concomitantly, the Yop-effector proteins are translocated across the plasma membrane of the eukaryotic cell while the bacterium remains at the surface of the cell. The translocation mechanism is polarized and is dependent on at least three proteins YopB, YopD and LcrV respectively. At least LcrV and YopD are localized on the bacterial surface prior to cell contact indicating that these two proteins are important in the early contact dependant events of Yop effector translocation. Our results suggest that theYopB, YopD and LcrV are involved in forming a pore in the membrane of the target cell. This has led us to suggest that the Yop-effectors are translocated through this pore. Interestingly YopD is also translocated, into the target cell. After translocation, the different Yop-effectors are targeted to different regions of the eukaryotic cell. The protein-tyrosine phosphatase YopH, showing high homology with eukaryotic PTPases, blocks immediate early phosphorylation signals after infection. YopH is targeted to focal-adhesions leading to disruption of these structures. We have recently identified a sequence of YopH that is essential for the localisation of YopH to focal adhesions. A *yopH* mutant lacking this region is affected in anti-phagocystosis and is avirulent although the corresponding YopH protein exhibits full PTPase activity and is translocated into hosts cells as efficient as the wild-type protein. This may provide an explanation to why pathogenic strains of *Y. pseudotuberculosis* can block uptake by eukaryotic cells.

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Salmonella has evolved the unique ability to engage the host cell in highly coordinated interactions. These interactions required the function of a specialized protein secretion system termed type III which directs the secretion and subsequent delivery into the host cell of a battery of bacterial effector proteins. The activity of these effector proteins results in the stimulation of marked actin cytoskeleton rearrangements and nuclear responses that lead to bacterial internalization and the production of pro-inflammatory cytokines. The stimulation of these responses is the result of a remarkable modulation of the function of small GTP-binding proteins of the Rho subfamily (CDC42, Rac and Rho) by bacterial effector proteins. Initially, the delivery into the host cells of the type III secreted protein SopE, which acts as a guanine nucleotide exchange factor, results in the stimulation of CDC42- and Rac-dependent signaling pathways. The cellular responses are subsequently reversed by the activity of another bacterial protein, SptP, which acts as a GTPase activating protein (GAP) for CDC42 and Rac. *Salmonella* also influence the actin cytoskeleton through the function of another bacterial effector, SipA, which binds actin effectively reducing its critical concentration (the concentration of actin required for polymerization), stabilizing F-actin and increasing the bundling activity of T-plastin.

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REGULATION OF VIRULENCE GENE EXPRESSION IN ENTEROPATHOGENIC *Escherichia coli*: NEGATIVE AND POSITIVE CONTROL OF THE TYPE III SECRETION GENES.

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EPEC pathogenesis is characterized by the formation of attaching and effacing lesions on the surface of epithelial cells. Secretion and translocation of EPEC virulence factors require the components of a type III secretion system encoded in at least three potential operons within the locus for enterocyte effacement (LEE). In this work, we have extended our studies on virulence gene regulation in EPEC by analyzing the molecular mechanisms underlying the transcriptional regulation of the *sepZ, orf12* and *orf1* operons, which encode the components of the type III secretion system. Using transcriptional fusions of different fragments spanning the intergenic region between *sepZ* and *orf12*, and primer extension analysis, we found that their expression is controlled by overlapping divergent promoters contained between these two genes. Interestingly, expression of a *sepZ-cat* fusion carrying upstream sequences including the *orf12* open reading frame and an *orf12escV-cat* fusion was highly reduced in an *E. coli* K12 derivative strain, indicating that a chromosomally-encoded EPEC-specific gene was required for their activation. Search for this activator allowed us to determine that the product of the *orf1* gene, located in the LEE locus, was required for *sepZ* and *orf12* activation. Expression of *sepZ* and *orf12*, but not *orf1*, was abolished in an *orf1* mutant derivative of EPEC. In addition, we observed that the *bfpTVW/perABC*, which encodes the positive activator of *bfpA* and *bfpT*, was not required for expressing either operon including *orf1*. However, expression of *orf1-cat* fusions could not be detected in *E. coli* K12, indicating that its expression requires an additional factor. Further analysis revealed that expression of shorter *sepZ-cat* and *orf12-cat* fusions, lacking most of the *orf12* structural sequence, was similar between EPEC wt and *E. coli* K12. This suggested that a negative regulatory sequence, controlling simultaneously the activation of both the *sepZ* and *orf12* promoters, was located within the first nucleotides of the *orf12* structural gene. Expression of *sepZ-cat* and *orf12-cat* fusions in different *E. coli* K12 derivatives carrying mutations in global regulators, showed that these divergently oriented promoters are negatively regulated by H-NS. The implications of these findings in the regulation of other EPEC virulence genes has also been studied and will be discussed in the context of this work.

ENVIRONMENTAL REGULATION OF *SALMONELLA* PATHOGENICITY ISLAND 2 EXPRESSION

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Salmonella spp. harbor two large pathogenicity islands encoding type III secretion systems. *Salmonella* Pathogenicity Island 1 (SPI1) is import for the invasion phenotype, triggering of cytoskeletal rearrangements and modification of host cell signal transduction. SPI2 is required for systemic infections and proliferation of *Salmonella* in infected host organs. Strains harboring mutations in genes encoding the type III secretion system or a two-component regulatory system encoded by SPI2 are highly attenuated in virulence and show reduced accumulation within infected host cells. It has been demonstrated that reporter fusions to SPI2 genes are specifically induced by intracellular *Salmonella*.

We set out to identify the environmental cues affected the expression of SPI2 genes *in vitro*. Single copy transcriptional fusions of SPI2 genes to luciferase were generated and used to quantity expression. In addition, antibodies were raised against recombinant SPI2 proteins and used to monitor the levels of SPI2 proteins under various growth conditions. Starvation for phosphate induced SPI2 expression, as well as growth in media with limiting concentrations of Mg^{2+} or Ca^{2+}. The effect of divalent cations indicated that PhoPQ, a global regulatory systems of *Salmonella* virulence genes, is involved in the modulation of SPI2 gene expression.

These analyses revealed that SPI2 is specifically induced under environmental conditions corresponding to the phagosomal lumen. Comparison of environmental cues indicated that expression of SPI1 and SPI2 is inversely regulated, thereby preventing the simultaneous expression of two type III secretion system within one bacterial cell.

OmpR/EnvZ REGULATES THE TWO-COMPONENT REGULATORY SYSTEM SsrA/B IN *SALMONELLA* PATHOGENICITY ISLAND 2

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Salmonella pathogenicity island 2 (Spi2) encodes a putative, two-component regulatory system, SsrA/B, which regulates a type III secretion system needed for replication inside macrophages and systemic infection in mice. The sensor/regulator homologs, *ssrA/B*, and genes within the secretion system, including the structural gene *ssaH*, are transcribed after *Salmonella* entry into host cells. We are studying the transcriptional regulation of *ssrA/B* and the secretion system using *gfp* fusions to the *ssrA* and *ssaH* promoters. We find that early transcription of *ssrA*, after entry into macrophages, is most efficient in the presence of OmpR/EnvZ. An *ompR* mutant strain does not exhibit replication within cultured macrophages. Furthermore, footprint analysis shows that purified OmpR protein binds directly to the *ssrA* promoter region. We also show that minimal media, pH 4.5 can induce Spi2 gene expression in wildtype, but not *ompR* mutant strains. We conclude that the type III secretion system of Spi2 is regulated by two sensor/kinase systems, one of which (OmpR/EnvZ) activates expression of the second (SsrA/B) soon after entry into macrophages.

THE EXPRESSION OF GENES ENCODING SECRETED PROTEINS IN *SALMONELLA TYPHIMURIUM* REQUIRES THE TRANSCRIPTIONAL ACTIVATOR INVF AND THE INVASION PROTEIN CHAPERONE SICA

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Invasion of the small intestine epithelium by *Salmonella* requires the expression of many genes, the majority of which are within *Salmonella* pathogenicity island 1 (SPI1) at centisome 63. Many of these genes encode components of a type III secretion apparatus and effector proteins secreted by this system. The secreted invasion/enteropathogenesis protein SigD (or SopB in *S. dublin*) encoded by *sigDE* at centisome 25.5 requires the SPI1-encoded system for secretion. However, it was not known if the lack of SigD from culture supernatants of a SPI1 apparatus mutant was due to a defective secretion apparatus or to decreased expression of *sigDE*. The expression of a *sigD-lacZYA* fusion has since been determined to be reduced in an *invA* polar mutant. In addition, a screen for mutants with reduced *sigD-lacZYA* expression identified a mTn*5*Cm insertion in *spaS*, another gene believed to encode part of the type III secretion apparatus. The expression of *lacZY* transcriptional fusions to other genes encoding secreted effectors was also reduced in the *spaS*::mTn*5*Cm background. These data initially suggested the presence of a negative feedback control system for the expression of *sigDE*. However, the mutation in *spaS* was complemented by *sicA*, and not *spaS*. This result suggested that the mTn*5*Cm insertion was probably polar on the expression of *sicA*, a gene which encodes a putative invasion effector chaperone. A disruption mutation in *sicA* abolished the expression of *sigD-lacZYA* as well as transcription of a *sicA-lacZYA* reporter. In addition to SicA, the activation of both promoters required InvF, a member of the AraC/XylS family of transcriptional activators. Surprisingly, the expression of both the *sigD* and *sicA* promoters could be activated by InvF and SicA in *E. coli*, suggesting that these two proteins are sufficient for the transcription of these genes.

THE *S. TYPHIMURIUM* TRANSLOCASE PROTEIN, SSPC, INSERTS INTO THE PLASMA MEMBRANE OF EPITHELIAL CELLS TO FACILITATE TRANSLOCATION OF BACTERIAL EFFECTOR PROTEINS TO THE HOST CELL CYTOSOL

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Upon contact with eukaryotic cells, *Salmonella* species translocate a number of bacterial effector proteins into the host cell cytosol via specialized Type III secretion systems (TTSS). This process requires an intact Type III secretion apparatus and at least three translocase proteins, SspB, SspC and SspD (also known as SipB, SipC and SipD). Although the exact functions of the translocase proteins have not yet been elucidated, it is likely that they interact directly with eukaryotic cells to allow translocation to proceed across the plasma membrane. Consistent with this hypothesis, our studies have shown that SspC is associated with both membrane and cytosolic fractions of epithelial cells within the first fifteen minutes of infection. One potential topology for SspC associated with membrane fractions suggests that the carboxy terminal domain is free to interact with cytosolic proteins. Using the yeast two hybrid screen, we have identified a potential interaction between SspC and the eukaryotic intermediate filament protein cytokeratin 8. Point mutations in the carboxy terminus of *sspC* disrupt this interaction in yeast. *Salmonella* strains expressing these point mutants are defective for invasion and translocation of a TTSS target, indicating that the two hybrid interaction with cytokeratin 8 is predictive for SspC function. Our results suggest a model in which SspC is inserted into the host cell membrane, thereby allowing the carboxy terminal domain to interact with intermediate filaments. We propose that this interaction functions as a molecular anchor to stabilize the translocation apparatus and facilitate subsequent translocation of bacterial effector proteins into the eukaryotic cell.

THE YERSINIA VIRULENCE FACTOR YOPJ INHIBITS ACTIVATION OF THE MITOGEN ACTIVATED PROTEIN KINASE KINASE SUPERFAMILY.

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The bacterial pathogen *Yersinia* uses a type III secretion system to inject several virulence factors into target cells. One of the *Yersinia* virulence factors, YopJ, binds directly to the superfamily of MAP kinase kinases (MKKs) blocking both phosphorylation and subsequent activation of the MKKs. Our results explain how YopJ can inhibit the ERK, JNK, p38 and NFkB signaling pathways, prevent cytokine synthesis and promote apoptosis. We propose that the YopJ-related proteins found in a number of bacterial pathogens of animals and plants function to block MKKs so that host signaling responses can be modulated upon infection.

The Role of Oxygen-Dependent Host Defenses in Salmonella Pathogenesis

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Macrophages employ two principal oxygen-dependent systems to inhibit or kill ingested microbes: the NADPH phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS). Studies in knock-out (ko) mice and macrophages deficient in either or both of these systems have demonstrated that both phox and iNOS play important roles in host defense against *Salmonella*. Measurement of reactive oxygen and nitrogen species generated by wild-type macrophages indicate that oxidative chemistry predominates early, followed by a prolonged nitrosative phase. The NADPH oxidase is required for rapid *Salmonella* killing by macrophages, and iNOS provides both synergy and a sustained bacteriostatic effect. Course of infection studies in phox ko mice show early enhancement of *Salmonella* proliferation, while studies in iNOS ko mice reveal a late increase in bacterial burden, mirroring the observations in macrophages. The use of defined *Salmonella* mutants in these experimental systems is providing novel insights into mechanisms of phox and iNOS-dependent antimicrobial actions, as well as relevant mechanisms of resistance.

ROLE OF NF-κB ACTIVATION IN HOST CELL SURVIVAL DURING RICKETTSIAL INFECTION AND DISEASE PROGRESSION IN MICE

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We have demonstrated that activation of the transcription factor, NF-κB , occurs in cultured endothelial cells and other cell types during intracellular infection with *Rickettsia rickettsii*. When this activation is blocked, either pharmacologically or using genetically-altered cell lines, apoptosis of the infected host cell rapidly ensues. Thus, rickettsia-induced NF-κB activation is required for host cell survival during infection. This finding led us to hypothesize that NF-κB activation of host cells is required during *in vivo* infection for establishment of disease, since apoptosis would eliminate the site of replication of this obligate intracellular parasite leading to clearance of the organism. Mice lacking the p50 subunit of NF-κB (p50-/-), which display no developmental phenotype, were infected with *R. australis,* which causes lethal infection in wild-type mice. Fibroblasts cultured from these mice exhibited no NF-κB activation in response to infection and the cultures underwent rapid and extensive apoptosis. Whereas wild-type mice exhibited severe vasculitis typical of rickettsial disease, p50-/- mice exhibited no overt outward signs of infection, and little histologic evidence of inflammation. Liver lesions containing apoptotic hepatocytes were seen in p50-/- mice but not in wild-type mice. Thus, NF-κB activation during rickettsial infection appears to be essential for establishment of disease, likely by thwarting rapid host cell loss by apoptosis.

PHENOTYPIC VARIATION AND INTRACELLULAR PARASITISM BY *HISTOPLASMA CAPSULATUM*

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Histoplasma capsulatum is a dimorphic fungal pathogen that grows in a saprophytic mycelial form or a parasitic yeast form. In its yeast phase, the organism is superbly adapted for survival and proliferation within mammalian cells. Our work has focused on two yeast-phase-specific phenotypes that have a suspected role in virulence: cell wall α-(1,3)-glucan and a secreted calcium-binding protein (CBP). We have observed that yeast cells modulate synthesis of α-(1,3)-glucan in response to cell density, analogous to bacterial quorum sensing. Loss of α-(1,3)-glucan has been correlated with loss of strain virulence for mice and macrophages, and we have used such strains in complementation cloning strategies to identify sequences that are linked to yeast expression of the α-(1,3)-glucan phenotype. The synthesis and secretion of CBP has been monitored with *CBP1-gfp* fusion plasmids during growth, dimorphic conversion, and intracellular parasitism, demonstrating the patterns of transcriptional activation. To evaluate the role of CBP, we have developed a two-stage gene disruption strategy that relies on allelic exchange with linear telomeric plasmids. The resulting *cbp1* strains are defective in virulence and also in the ability to grow in limiting calcium conditions. This establishes CBP as the first genetically proven virulence determinant of *H. capsulatum* and suggests a link between intraphagosomal calcium levels and intracellular survival.

SYSTEMATIC IDENTIFICATION OF CLASS-I ACCESSIBLE PROTEINS IN SALMONELLA TYPHIMURIUM

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Intracellular bacterial pathogens adapt to their host cell environment by the selective secretion of proteins designed to alter the normal structural and metabolic machinery of the host cell, thus promoting bacterial survival and avoidance of host immune surveillance. Many bacterial proteins are known to mediate their effects within the host cell cytoplasm. Because access to the cytoplasm of the host cell also infers access to the degradative machinery of the host cell proteosome, we have collectively labeled these proteins **C**lass I (MHC, HLA) **A**ccessible **P**roteins (CAPs). Whole genome analysis has vastly increased our knowledge of the genetic nature of many different pathogens, however, this knowledge is of limited value in the assignment of function to proteins encoded by bacterial genes with no known homology. The analysis of host interaction is made difficult by the complex nature of the pathogenic lifecycle of intracellular pathogens such as Salmonella. The identification of CAPs secreted in response to host cell invasion, would be an invaluable resource for the study of host response to infection. In addition, since CAPs possess unique access to the host's antigen processing and presentation machinery, they represent potentially attractive vaccine targets and useful vehicles for the delivery of foreign epitopes by bacterial carrier vaccines. To identify CAPs in *Salmonella typhimurium*, we developed a novel approach, termed "**D**isseminated **I**nsertion of **C**lass I **E**pitopes (DICE), which uses a resolvable Tn5-based transposon to randomly distribute the H-2K^b-restricted ovalbumin epitope, SIINFEKL, throughout the bacterial chromosome. When the resolved insertion is in-frame within a gene, CAPs released from the infecting bacteria are processed by the proteolytic machinery of the host cell, resulting in the presentation of the carried ovalbumin epitope SIINFEKL, in the context of H-2K^b. *Salmonella* strains containing SIINFEKL insertions within CAPs are then easily isolated by flow cytometry.

UNIQUE CELL WALL-ASSOCIATED LIPID REQUIRED FOR TISSUE-SPECIFIC REPLICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN MICE

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Tuberculosis (TB) is the leading cause of death in the world due to a single bacterial infection. Despite its enormous burden on world health, little is known about the molecular mechanisms of *M. tuberculosis* pathogenesis. Bacterial multiplication and concomitant tissue damage within an infected host occur primarily in the lungs - the favored niche of *M. tuberculosis*. Although it has been postulated that the distinctive cell wall of *M. tuberculosis*, rich in a variety of unique lipids and glycolipids, is important for virulence of the organism, rigorous genetic proof has been lacking. Using signature-tagged mutagenesis, we have isolated *M. tuberculosis* mutants that fail to replicate normally within the lungs of infected mice. Interestingly, three such mutants are unable to synthesize or transport a complex, cell wall-associated lipid known as dimycoserosalphthiocerol (DIM). Two of these mutants have transposon insertions that affect genes encoding polyketide synthase-like enzymes which are required for DIM synthesis. The third mutant has a disruption in a gene encoding a large transmembrane protein that is required for proper secretion of the lipid to the cell surface and into the culture media. Surprisingly, production and transport of DIM is only required for growth in the lung; all three mutants are unaffected for growth in the liver and spleen. Therefore, we believe that the DIM biosynthetic pathway is present to protect *M. tuberculosis* from a lung-specific defense mechanism of the host. Furthermore, we consider that an *M. tuberculosis* mutant unable to synthesize DIM could play an important role in the development of a superior replacement for the currently used BCG vaccine.

INTERACTION OF MATURE *LEGIONELLA* REPLICATION VACUOLES WITH THE MACROPHAGE ENDOCYTIC PATHWAY.

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It is well established that after phagocytosis by macrophages, the Gram-negative bacterium *Legionella pneumophila* inhibits fusion of its phagosome with lysosomes, then begins replication in association with the endoplasmic reticulum. Because little is known about the dynamics of the mature replication vacuole, we examined by fluorescence microscopy the interactions between the endocytic network and *L. pneumophila* vacuoles as they aged. During the first 8 h period, virulent *L. pneumophila* remained in lag phase within nonfusigenic vacuoles, but by 12 h bacterial replication had resumed. Surprisingly, during the 12 to 30 h period of the primary infection, a significant proportion of *L. pneumophila* replication vacuoles acquired endosomal characteristics. Firstly, membranes of 70% of the replication vacuoles were positive for LAMP-1, and the lumen of 50% of these compartments contained cathepsin D. Secondly, up to 50% of the replication vacuoles had acquired Texas Red-ovalbumin which had been preloaded into the lysosomal network. Finally, Texas Red-dextran endocytosed by infected macrophages was delivered to these *L. pneumophila* compartments with similar kinetics as delivery to *E. coli* phagolysosomes.

Interestingly, acquisition of endocytic markers by *L. pneumophila* vacuoles had no apparent detrimental effect on bacterial growth: during the 12 to 30 h infection period, the yield of CFUs typically increased 10-fold. Moreover, inhibition of vacuole fusion with the endocytic network by bafilomycin resulted in arrested replication of intracellular *L. pneumophila*, an effect which was reversible upon drug removal.

Presently, our experiments focus on the mechanism of this switch from a non-fusigenic pathogen vacuole to one with endocytic characteristics. Virulent post-exponential *L. pneumophila* which have been diluted into broth also lose the ability to inhibit phagosome-lysosome fusion after 10 to 12 h, kinetics similar to that of fusion and growth observed after *L. pneumophila* enter macrophages. Thus, our data indicate that when cultured in macrophages, as in broth, only post-exponential phase *L. pneumophila* express the factor(s) that blocks fusion of lysosomes with the nascent phagosome; when bacterial replication resumes, the *L. pneumophila* vacuole interacts with the endocytic pathway, a potential source of nutrients.

IDENTIFICATION OF GENES REQUIRED FOR CHRONIC INFECTION BY *BRUCELLA ABORTUS* USING SIGNATURE-TAGGED MUTAGENESIS R.M. Tsolis, P.C. Hong, and T.A. Ficht. Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A & M University, College Station, TX 77843-4467

Long term survival and growth within host macrophages is a unique bacterial lifestyle shared by many important human pathogens. The mechanisms of intracellular persistence are, for the most part, unknown. *Brucella abortus*, a facultative intracellular pathogen that causes chronic infections in humans, can be used to study intracellular persistence because it lends itself to genetic analysis. The ability of *B. abortus* to establish chronic infections in a host is attributed to its long-term survival and growth within macrophages of the host. In order to study the genetic basis of intracellular persistence, we used signature-tagged transposon mutagenesis (STM) to screen for random insertional mutations that affect ability of *B. abortus* to cause persistent infection in mice. 240 STM mutants were screened at two different time points post-infection. The ability to establish infection in the mouse was assessed by recovering mutants from the spleen at two weeks post-infection, and the ability to cause chronic infection was assessed at eight weeks post-infection. Mutants were grouped into those which showed a delay in colonization (absent at two weeks, but present at eight weeks), those which failed to colonize (absent at both time points), and those which colonized but were unable to persist in the host (present at two weeks, but absent at eight weeks). After confirmation of the colonization defect by competitive infection, we found that 7% of the mutant pool are unable to establish chronic infection. Sequence analysis revealed that some of the genes required for intracellular persistence are related to genes involved in host-pathogen interactions of the closely related genera *Rhizobium* and *Agrobacterium*. We are currently examining the role of these genes in interactions with host phagocytic cells.

GENETIC ANALYSIS OF *Salmonella* PERSISTENCE WITHIN EUCARYOTIC CELLS

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NRK rat fibroblasts and other non-phagocytic eucaryotic cell lines arrest *Salmonella typhimurium* intracellular proliferation. Within these cells, intracellular bacteria remain in a latent stage and maintain viability up to 7 days. We have used this *in vitro* model to identify bacterial determinants involved in the persistent infection phenotype. Two processes underlying intracellular latency of *S. typhimurium* have been differentiated. First, a pathogen-mediated self-restriction for intracellular growth; and, second, an adaptation mechanism linked to bacterial persistence within the host cell.

Genetic analysis shows that the two-component regulatory system PhoPQ is required for self-restriction of intracellular growth: PhoP$^-$ mutants have an increased intracellular growth rate within NRK cells while intracellular wild-type bacteria do not proliferate. Overproduction of PhoP protein in an PhoP$^-$ mutant exacerbates bacterial death within these cells, suggesting that a proper amount of PhoP protein is required for both self-restriction of bacterial growth and entrance in a further persistence stage. Evaluation of *phoP*-regulated genes with a potential role in these phenotypes indicate that the *pmrA*-regulon might be partially involved. Support to this observation is provided by the fact that, unlike intracellular wild-type bacteria, a *phoP* mutant does not vary the length of its lipopolysaccharide (LPS) O-chains when residing in intracellular locations.

Genetic characterization of bacterial isolates rescued from 7-day-infected NRK cells show a high frequency of mutant variants which have a slow growth rate in laboratory conditions. Mutations have been mapped in genes related to respiratory metabolism, as *lpd* (encoding for lipoamide-dehydrogenase), *hemL* (aminolevulinate synthase) and *aroD* (transporter of aromatic aminoacids). Our working hyphotesis is that *S. typhimurium* may adapt to persistence conditions by switching off respiratory metabolism, thus avoiding damage caused by host-derived and endogenous oxygen-reactive compounds. These "persistent" mutants have shown to be attenuated in the BALB/c mouse model, reinforcing the relevance of pathogen persistence without concomitant host killing.

SERINE PROTEASE AUTOTRANSPORTER TOXINS OF *E. coli* AND SHIGELLA

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Many Gram-negative pathogens secrete toxigenic proteins into the external milieu. A rapidly growing class of secreted proteins includes the autotransporter proteins, which utilize the outer membrane transport mechanism originally characterized for the IgA protease of *Neisseria gonorrhoeae*. Diarrheagenic *E. coli* and *Shigella* strains secrete a subfamily of autotransporter proteins which feature serine protease motif signatures near the N-terminus of the mature protein. The roles of the serine protease domains, and indeed of the proteins themselves, have not been fully characterized for any enteric species. We have described two secreted serine proteases of *E. coli* and *Shigella* with distinct features. One, designated the plasmid encoded toxin (Pet) from enteroaggregative *E. coli* (EAEC), is capable of causing cytoskeletal disruption of epithelial cells in culture and also of eliciting rises in short circuit current in intestinal tissue mounted in the Ussing chamber. This toxin is prototypical of a subfamily of related toxins in other enteric pathogens. Our evidence suggests that Pet acts as a protease at an intracellular target. A second prototype protease, designated Pic from *S. flexneri* and EAEC, is a mucinase and is essential for the induction of inflammatory and invasive effects of the parent *Shigella* strain in the rabbit ileal loop. Pic may also play a role in resistant to serum complement. The secreted serine protease autotransporters represents a highly conserved family of proteins which may serve virulence functions in a variety of enteric pathogens.

HOST GENES IN PATHOGEN RESISTANCE: ROLES FOR HUMAN OSTEOPONTIN IN MYCOBACTERIAL INFECTION

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We have developed a general strategy to identify candidate genes involved in host resistance to pathogens by analyzing host cell gene expression changes after infection. Genes identified by a screening process are tested within a model system and then evaluated in clinical specimens. Using this approach, the effects of mycobacterial infection on macrophage gene expression were studied to understand the pathogenesis of tuberculosis.

Differential screening of a macrophage cDNA library repeatedly identified one gene whose expression increased after infection by mycobacteria. This gene encodes a protein called osteopontin: a phosphoprotein secreted from macrophages, T cells, and NK cells and a known macrophage chemoattractant. An abundance of osteopontin protein was confirmed in human pathologic specimens of tuberculosis implicating it in the host response against *Mycobacterium tuberculosis*. Osteopontin null mice challenged by infection with *M. bovis* BCG had delayed clearance of the BCG and more extensive granulomatous inflammation compared to wildtype animals. BCG grew more rapidly in macrophages derived from osteopontin null mice versus those from wildtype mice, demonstrating the null phenotype was due to an intrinsic macrophage defect. Patients with interferon-γ receptor deficiencies or idiopathic immune defects who have disseminated infection after BCG vaccination had no significant osteopontin accumulation in infected lymph nodes. Normal patients with regional adenitis or a patient with IL-12 receptor deficiency, who all had good clinical outcomes, had abundant osteopontin accumulation. Thus, tissue expression of osteopontin correlates with effective immune and inflammatory responses when humans are challenged by a mycobacterial infection and likely contributes to human resistance against disease.

Our findings demonstrate that surveying normal cell gene expression provides useful information on the mechanisms of resistance to infection. We are expanding this analysis by using gene arrays to identify human genes responsive to infection by several bacterial and viral pathogens.

CHARACTERIZATION OF EPEC ISOLATES FROM A COLONY OF COMMON MARMOSETS EXPERIENCING ACUTE WATERY DIARRHEA.

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Diarrhea is an important cause of morbidity and mortality in non-human primates. Common marmosets (*Callithrix jacchus*) were housed at the New England Regional Primate Research Center (NERPRC). These animals developed acute watery diarrhea with hematochezia and dehydration. Fecal cultures and biopsies were performed within 24 hours from the onset of clinical signs.

H&E stained sections of colonic tissue revealed rows of bacteria adhered diffusely or multifocally along the brush border of surface epithelium. These changes were accompanied by crypt hyperplasia, congestion and increased numbers of neutrophils within the lamina propria. Electron microscopic examination of biopsy specimens showed bacteria attaching to colonic epithelium by distinct pedestal formation and effacement of microvilli (AE lesions).

Escherichia coli strains were isolated from the affected monkeys and screened for the presence of *eaeA* and *espB*, two genes shown to be necessary for intimate attachment and signal transduction between EPEC and host enterocytes, by PCR and Southern hybridization. Clinical isolates 961185 and 961193, obtained from animals Cj8793 and Cj31195 respectively, were positive for the presence of both *eaeA* and *espB*. The *E. coli* isolated from these animals were negative for shiga-like toxin I and II both by PCR and Southern hybridization.

A library of clones derived from one of the two marmoset isolates was probed, and an 8-kb fragment was isolated which contains the entire *eaeA* gene. Sequence analysis of the C-terminal portion of the predicted protein shows it to be more homologous to EPEC strain E2348/69 than to EHEC strain EDL932. We are currently characterizing additional virulence factors present in these isolates and constructing nonpolar insertional mutations in the LEE for use in investigating the utility of these strains in a non-human primate animal model for EPEC infection.

DIHYDROLIPOAMIDE ACETYLTRANSFERASE IS A CANDIDATE IN MEDIATING ADHESION TO *EX VIVO* BIOMATERIALS IN *STAPHYLOCOCCUS EPIDERMIDIS*

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Shot gun phage display has previously been used in our research group to isolate genes encoding adhesins in Gram-positive cocci. The use of a new generation of phagemid vectors makes it easier to identify binding activities when a phage display library is panned against complex mixures of ligands. *Staphylococcus epidermidis* is often associated with biomaterial related infections. In this study a phage display library of chromosomal DNA from a clinical strain of a *S. epidermidis* (strain 19) was panned against *ex vivo* screws, isolated from a patient with an operated bone injury. The library was also panned against pieces of an *ex vivo* central venous catheter (CVC). After the second panning colonies were isolated and the inserts of the phagemids were DNA-sequenced. In the case of the CVC-panning phagemids harbouring overlapping inserts from two distinct genes where found. One of them were *fbe,* a gene encoding a cell surface protein mediating adherence of *S. epidermidis* cells to immobilised fibrinogen (M. Nilsson, L. Frykberg, J-I. Flock, M. Lindberg, and B. Guss, Infect. Immun. Vol 66. 6:2666-2673, 1998). More surprisingly was that both in the panning against the CVC pieces and the screws phagemids harbouring overlapping inserts dominated of a gene homologous to the dihydrolipoamide acetyltransferase a component of the pyruvate dehydrogenase complex in *Staphylococcus aureus*. In order to analyse if the acetyltransferase is located on the cell surface in *S. epidermidis*, antibodies against the protein were raised in rabbit. Cell surface proteins of strain 19 was isolated by boiling a cell suspension in a sample buffer and after centrifugation the supernatant was added on a SDS-PAGE. In Western blot analysis a band with expected size could be detected by using peroxidas-conjugated anti-rabbit antibodies. ^{125}I-labelled anti-acetyltransferase antibodies were used to measure the expression of the acetyltransferase on the cell surface during growth in a broth culture. The result showed in peak in binding of antibodies in the early exponential growth phase. At present we are trying to isolate and identify the acetyltransferase interacting component(s) on the *ex vivo* biomaterials.

VIBRIO CHOLERAE H-NS SILENCES MULTIPLE VIRULENCE GENE PROMOTERS IN THE TOXR REGULON

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Vibrio cholerae pathogenesis requires the elaboration of a number of virulence factors, including cholera toxin (CT) and the toxin coregulated pilus (TCP). The genes for CT (*ctxAB*) and TCP (*tcpA-J*) are located on separate pathogenicity islands of phage origin. Expression of these genes, and those encoding related factors, is regulated by environmental conditions via a complex regulatory cascade known as the ToxR virulence regulon that involves both chromosomal and pathogenicity island encoded regulators. During growth under ToxR-expressive conditions, the *ctxAB* and *tcpA-J* genes are directly activated by the AraC homolog, ToxT. For this event to occur, the *toxT* gene must first be activated by TcpPH and ToxRS. Expression of *tcpPH* is itself under the control of additional regulatory proteins whereas the expression of *toxRS* is essentially constitutive. In this study we have determined that the abundant histone-like nucleoid structural protein H-NS silences ToxR virulence regulon genes. Deletion of *hns* results in constitutive, maximal expression of the *ctxAB*, *tcpA-J*, *toxT* and *tcpPH* promoters under all environmental conditions. The finding that high-level expression from *ctxAB*, *toxT*, and *tcpPH* also occurs in the absence of their cognate activators suggests that H-NS directly silences expression from these promoters. Furthermore, deletion analysis suggests that the interaction of H-NS at the *toxT* promoter includes a region upstream of the ToxRS and TcpPH binding sites. These results raise the possibility that one of the roles of the specific transcriptional activator proteins in the ToxR virulence regulon is to negate the repressive action of H-NS under various environmental conditions.

INTERACTIONS OF THE ANAEROBIC PATHOGEN *CLOSTRIDIUM PERFRINGENS* WITH MACROPHAGES UNDER AEROBIC AND ANAEROBIC CONDITIONS.

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Clostridium perfringens is a common cause of gas gangrene (clostridial myonecrosis), an invasive and lethal disease in humans and animals. When ischemic tissues are contaminated with *C. perfringens*, the bacteria multiplies rapidly and spreads into and destroys healthy tissue through the secretion of potent cytotoxins and degradative enzymes. The following experiments were designed to determine what capabilities the host innate immune system (e.g., phagocytic cells) has in ischemic tissues and how *C. perfringens* can evade these defense mechanisms in the early stages of the disease. We used the mouse J774A.1 macrophage-like cell line as a model system. The J774A.1 cells were able to survive for up to 48 h under completely anaerobic conditions, during which they induced the synthesis of LDH, a glycolytic enzyme. When *C. perfringens* cells were added to the macrophages under anaerobic conditions, the bacteria were phagocytized but grew exponentially and were cytotoxic to the macrophages. Transmission electron microscopy (TEM) of infected macrophages showed tightly encapsulated, intact bacteria internal and external to degraded macrophages and cell debris. Pre-activation of the macrophages with interferon-γ (IFN-γ) was shown to partially repress the cytotoxic effects of *C. perfringens*. Under aerobic conditions, *C. perfringens* cells were phagocytized but the bacteria were not killed, even if the macrophages were pre-activated with IFN-γ. TEM of infected macrophages under aerobic conditions showed intact macrophages with bacteria in spacious phagosomes. Analyses of phagosome-lysosomal fusion were performed using antibodies to the late endosome-lysosomal marker protein LAMP-1 and to whole bacterial cells using fluorescence microscopy. Under anaerobic conditions, there appeared to be little co-localization of the bacteria with the lysosomal marker, suggesting *C. perfringens* may be able to inhibit phagosome-lysosome fusion. Interestingly, heat killed bacteria were not phagocytized under anaerobic conditions, but were under aerobic conditions. This suggests there may be different mechanisms of bacterial uptake and that *C. perfringens* may play an active role in the process under anaerobic conditions. Currently, we are in the process of developing an intracellular survival assay to determine if *C. perfringens* can replicate within the phagosome under aerobic and anaerobic conditions.

A NEW ANTI-*M. AVIUM* MECHANISM ASSOCIATED WITH INDUCTION OF APOPTOSIS AND DEPENDENT ON MACROPHAGE ACTIVATION AND ENDOGENOUS PRODUCTION OF OXYGEN RADICALS.

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Tryptophan metabolism plays a role in immune mechanisms. During the activation of macrophages, the enzyme involved in the first step of tryptophan degradation , 2,3-indoleamine dioxygenase, is induced by IFN-γ. Picolinic acid (PA) is a terminal catabolite of L-tryptophan degradation. *In vitro*, PA was found to synergise with IFN-γ for the induction of tumoricidal activity in macrophages by increasing TNF-α mRNA expression and NO_2^- production. We have previously shown that PA plus IFN-γ induced complete bacteriostasis of *M. avium* in infected macrophages. The mycobacterial growth inhibition induced by PA and PA+ IFN-γ were associated with apoptosis of macrophages. Simultaneous addition of PA and IFN-γ increased the percentage of apoptotic cells as compared to PA treatment alone. We have now shown that apoptosis and bacteriostasis induced by PA± IFNγ are reduced by the prostaglandin 15d-PGJ_2 that binds to the peroxisome proliferator-activated receptor-γ (PPAR-γ). Furthermore, previous treatment with the anti-oxidant N-acetyl cystein (NAC) delays apoptosis and abrogates anti- *M. avium* activity induced by PA and PA plus IFN-γ. Since it has been demonstrated that activation of PPAR-γ inhibits the expression of genes up-regulated by IFN-γ by antagonizing the activities of transcription factors, we speculate that the induction of apoptosis and of anti- *M. avium* activity by PA± IFNγ requires gene transcription. On the other hand, this apoptotic process seems to be dependent on oxygen radicals that can either induce apoptosis by damaging or by acting as second messengers. We also suggest that the apoptotic machinery is able to induce bacteriostasis since the two effects cannot be dissociated with the treatment used here and it cannot be reverted by inhibition of NO synthesis, TNFα activity or scavenging of oxygen reactive intermediates.

CHARACTERIZATION OF A TYPE IV PILUS OF *VIBRIO VULNIFICUS*

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Vibrio vulnificus is capable of causing primary septicemia through the consumption of raw oysters, or wound infections through contact with contaminated water or shellfish. The fatality rate for primary septicemia in susceptible individuals is 50 to 75%, while wound infections are fatal for 20% of those affected. Specific virulence factors have been difficult to identify in *V. vulnificus*. Different strains of the organism exhibit varying virulence in animal models and patients with clinical septicemia usually harbor a single strain. However, other than virulence, clinical and environmental isolates are phenotypically indistinguishable.

Pili have been observed on the surface of some strains of *V. vulnificus*. One type, type IV, have been identified as virulence factors in several Gram-negative pathogens, because of their role in adherence and colonization. Previously, we have isolated and characterized two genes homologous to type IV pilus biogenesis genes, *vvpC* and *vvpD*. A mutation in *vvpD*, the gene encoding the type IV prepilin peptidase of *V. vulnificus*, abolishes expression of surface pili, blocks secretion of several exoenzymes that follow the type II secretory pathway, and decreases virulence. In this study, we report the isolation of the remaining genes in this type IV pilus biogenesis gene cluster (*vvpA* and *vvpB*), located immediately upstream of *vvpC* and *vvpD* and organized similarly to gene clusters from *Aeromonas hydrophila* (*tapABCD*) and *V. cholerae* (*vcp* or *pilABCD*). Cloning and sequencing of *vvpA* shows that it encodes a typical type IV pilin subunit gene, homologous to pilins from *Pseudomonas aeruginosa* (PilA) and *Vibrio cholerae* (MshA or PilA). The gene *vvpB* encodes a homolog of *Pseudomonas aeruginosa* PilB, the type IV pilus biogenesis accessory factor that is a member of the ABC transporter family.

To determine the distribution of the pilus biogenesis genes in *V. vulnificus* isolates, clinical and environmental isolates (from oysters) were compared for the presence of *vvpA* and *vvpD*. Although the different strains exhibit considerable polypmorphism in the location of these genes on the *V. vulnificus* chromosome, *vvpA* and *vvpD* are highly conserved in both clinical and environmental isolates. This suggests that type IV pili may play an important role in pathogensis as well as in persistence of the organism in its natural environment. Amino acid sequence comparison of VvpA from different isolates shows that *V. vulnificus* expresses a limited number of antigenically distinct type IV pilins.

BACTERICIDAL EFFECTS OF HYDROGEN PEROXIDE PRODUCTION BY *STREPTOCOCCUS PNEUMONIAE* ON OTHER INHABITANTS OF THE RESPIRATORY TRACT

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Streptococcus pneumoniae is a common inhabitant of the nasopharynx in humans and is the leading cause of meningitis, septicemia, and community-acquired pneumonia. An inverse correlation between colonization of the nasopharynx by *S. pneumoniae* and *Haemophilus influenzae*, another common upper respiratory pathogen, has been reported. Studies were undertaken to determine if either of these organsims produces substances which inhibit growth of the other. Culture supernatants from *S. pneumoniae* caused zones of inhibition when added to lawns of *H. influenzae*, whereas supernatant from *H. influenzae* had no effect on growth of *S. pneumoniae*. Additionally, coculture of *S. pneumoniae* and *H. influenzae* led to a decrease in viable *H. influenzae* to undetectable levels within several hours. The inhibitory activity of *S. pneumoniae* supernatant was not affected by proteinase K or heat treatment, suggesting that the inhibitory substance was not a protein. The addition of purified catalase, which specifically degrades hydrogen peroxide, prevented killing of *H. influenzae* in coculture experiments, suggesting that hydrogen peroxide may be responsible for this activity. A catalase-reversible inhibitory effect of *S. pneumoniae* on the growth of the respiratory tract pathogen *Moraxella catarrhalis* was also observed. *S. pneumoniae* is known to produce substantial quantities of hydrogen peroxide through the action of pyruvate oxidase (*spxB*) under conditions of aerobic growth. *S. pneumoniae* exhibited approximately 100 fold greater survival than *H. influenzae* when exposed to concentrations of hydrogen peroxide similar to that produced by *S. pneumoniae*. A positive correlation was noted between the availability of oxygen and the degree of bactericidal activity, consistent with the hypothesis that aerobic conditions are required for the production of hydrogen peroxide in *S. pneumoniae*. A naturally occurring variant of *S. pneumoniae* which is down-regulated in *spxB* expression and produces little or no hydrogen peroxide was unable to kill *H. influenzae*. The mechanism by which a catalase-negative organism is able to survive concentrations of hydrogen peroxide that are lethal for catalase-positive organisms is unclear. High level hydrogen peroxide production, therefore, may be a means by which *S. pneumoniae* is able to inhibit a variety of competing organisms in the aerobic environment of the upper respiratory tract.

BVG-INDEPENDENT *BORDETELLA PERTUSSIS* TON SYSTEM AND VIRULENCE

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In Gram-negative bacteria, high affinity iron uptake requires the TonB/ExbB/ExbD envelope complex to release iron chelates from their specific outer membrane receptors into the periplasm. Based on sequence similarities, the *Bordetella pertussis tonBexbBexbD* locus was identified on a cloned DNA fragment. The tight organization of the 3 genes suggests that they are cotranscribed. A putative Fur-binding sequence located upstream from *tonB* was detected in a Fur titration assay, indicating that the *tonBexbBexbD* operon may be Fur-repressed in high-iron growth conditions. Putative structural genes of the β-subunit of the histone-like protein HU and of a new two-component regulatory system were identified upstream from *tonB* and downstream from *exbD*, respectively. A *B. pertussis ΔtonBexbB*::Km^r mutant was constructed by allelic exchange and characterized. The mutant was impaired for growth in low-iron medium in vitro, and could not use ferrichrome, desferal, nor hemin as iron sources. Expression of the bacterial adhesins and toxins such as FHA, PRN, PTX, or AC-Hly was similar in the $TonB^+$/$TonB^-$ pair and still responsive to chemical modulators, indicating that the BvgA/BvgS virulence regulatory system is not TonB-dependent. However, in the murin respiratory infection model, the mutant ability to colonize the respiratory tract of the mouse was greatly reduced compared to the wild-type strain, suggesting a defect in its capacity to grow in the host.

THE VIRULENCE REGULATOR TOXR MEDIATES RESISTANCE TO BILE IN *VIBRIO CHOLERAE*

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Resistance to physiological concentrations of bile in the intestine is a prerequisite for virulence of enteric pathogens such as *Vibrio cholerae*. Minimal bacteriocidal concentrations (MBCs) of bile, the anionic bile detergent deoxycholate (DC) and sodium dodecyl sulfate (SDS) were determined for *Δ toxR*, *Δ toxT*, *Δ tcpP* mutant strains and wild type *Vibrio cholerae* to recognize a potential role in bile resistance for transcriptional activators known to induce expression of virulence factors. *Δ toxR* mutants derived from either El Tor E7946 or Classical 0395 strains showed consistently lower MBCs than the respective *Δ toxT*, *Δ tcpP* and wild type strains. Transformation with a $toxR^+$ vector restored wild type levels of resistance to bile, DC, and SDS in the *Δ toxR* mutants of either biotype. Likewise, *Δ toxR* mutants grown in various concentrations of DC or SDS displayed defective growth kinetics compared to wild type and *Δ toxT* mutant strains. Total protein patterns resolved by SDS-PAGE of wild type, *Δ toxT*, and *Δ tcpP* mutant strains grown in LB supplemented with bile or DC revealed overexpression of a protein migrating at 38 kDa which was absent from Δ*toxR* strains. Purification of outermembrane fractions of *V. cholerae* grown in the presence of bile demonstrated increased accumulation of a 38 kDa protein in the membranes of wild type but not *Δ toxR* mutants derived from either biotype, and this protein was confirmed to be the outer membrane porin OmpU by Western blot. β-galactosidase activity of *ompU-lacZ* promoter fusions introduced into wild type, *Δ toxT* and *Δ toxR* mutant strains confirmed that *ompU* transcription was dependent on the presence of ToxR. However, ToxR-dependent transcription of *ompU* was not further stimulated by the presence of bile or DC, suggesting possible post-transcriptional mechanisms for increasing levels of OmpU in the outer membrane. These results are consistent with a role for OmpU or another ToxR-dependent, ToxT-independent mediator of bile resistance in both *Vibrio cholerae* biotypes.

THE OPPORTUNISTIC PATHOGEN *PSEUDOMONAS AERUGINOSA* KILLS *DICTYOSTELIUM DISCOIDEUM*: A NEW GENETIC MODEL FOR STUDYING HOST/PATHOGEN INTERACTIONS?

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Microbial pathogens have evolved in relation to their mammalian hosts and have developed strategies for colonization, invasion, and pathogenesis. These infections are highly specific, involving specific host targets and signaling pathways. Some of these strategies involve the induction of host responses to facilitate the growth of the pathogen. In this case, the host becomes an active participant in the infection process.

One strategy to study the interactions between a pathogen and its host is the use of genetically tractable host models to study infections by identifying resistant host mutants. This approach may aid us in understanding the molecular mechanisms. With this in mind, we tested whether *Dictyostelium* could be used as a host model for the opportunistic pathogen *P. aeruginosa*. This gram-negative bacterium produces an array of secreted virulence factors during an infection. Although our immune system is equipped to fight such infection, individuals with a compromised immune system are at great risk of life-threatening *Pseudomonas* infections.

Incubation of *P. aeruginosa* with *Dictyostelium* cells in suspension or on agar plates leads to the lysis of *Dictyostelium* cells. In order to determine whether this interaction is based on a known virulence factor, we tested avirulent strains of *Pseudomonas* (courtesy of Fred Ausubel, Harvard University) for their virulence towards *Dictyostelium*. We identified one mutant that is unable to kill *Dictyostelium*. In fact, *Dictyostelium* feeds on this avirulent mutant, which carries an insertional mutation in the *lasR* gene. LasR is a transcription factor that is responsible for the expression of an array of virulence factors. *lasR* is induced at high population densities and this density or quorum sensing is an important regulator of pathogenesis.

We suggest that *Dictyostelium* can be used as a host to study the mechanisms of *Pseudomonas* infection. *Dictyostelium* must employ sophisticated strategies to utilize pathogenic bacteria as a source of nutrients, mechanisms that might be conserved in human cells.

INFECTION OF MONOCYTES BY *M. TUBERCULOSIS* REGULATES EXPRESSION OF IRF-1, A KEY IMMUNOMODULATORY TRANSCRIPTION FACTOR.

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IRF-1 is a transcription factor that has a pleiotropic role in innate and adaptive immunity. Transcription of the IRF-1 gene is activated by many cytokines, including interferons (IFNs) and tumor necrosis factor α (TNFα). Studying the regulation of IRF-1 expression and the interaction of IRF-1 with the genes it regulates is critical to understanding its function in immunity and inflammation. A typical, predominant complex of IRF-1 with DNA *in vitro* is detected by electrophoretic mobility shift assay (EMSA) of extracts from cells treated with IFNα or TNFα. When IRF-1 is present at high concentrations, for example, after induction by IFNγ, an additional larger complex forms. The typical complex and the larger complex can be formed by purified or recombinant full length IRF-1. These observations, made initially during purification of IRF-1 from HeLa cells, were also true for B lymphoid (Daudi), and T lymphoid (Jurkat) cell lines, among others. However, in a human monocytic cell line (THP-1), IRF-1 induced by either IFNα or IFNγ formed multiple complexes of greater mobility than those previously seen. SDS-PAGE and western blot analysis revealed that apparently small forms of IRF-1 protein were induced in the monocytic cells. Thus, detection of rapid mobility IRF-1/DNA complexes correlated with the presence of the novel forms of IRF-1 protein. After activation of monocytes by infection, or after monocyte to macrophage differentiation, the typical IRF-1/DNA complex and full length IRF-1 protein were induced upon treatment with IFNα or IFNγ, while induction of the truncated forms of IRF-1 and detection of small complexes were reduced or eliminated. To determine the precise correspondence between the IRF-1/DNA complexes and the IRF-1 protein species, protein/DNA complexes were resolved by EMSA, lanes from the native gel were placed across the top of SDS-PAGE gels, the proteins in each complex were resolved, and the IRF-1 species that had been in each protein-DNA complex were detected by western blot. This 2D analysis showed that each rapid mobility complex contained a particular small form of IRF-1, and that the typical complex contained full-length IRF-1. The switch in IRF-1 expression from truncated species to the full length form correlated with greater interferon-inducibility of genes regulated by IRF-1. (Supported by NIH grant 5 RO1 AI37877 and by a grant from the Arthritis Foundation.)

USE OF RECOMBINANT NONCONJUGAL *Escherichia coli* VECTOR CARRYING *ereB* GENE IN RICKETTSIAL TRANSFORMATION

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Members of the genus *Rickettsia*, contains several well-known human pathogens, such as *R.typhi* and *R. akari*, the etiologic agents of endemic typhus and rickettsialpox. Transformation of *Rickettsia* is a recent accomplishment, but utility of this technique is limited due to the paucity of selectable markers. A real challenge facing rickettsiologists is to introduce certain gene into rickettsial chromosome where there is no DNA sequence homology. Recently, we cloned and characterized a hemolysin gene, *tlyC* from *R. typhi*. The *R. typhi tlyC* gene was introduced into a recombinant nonconjugal *E.coli* vector (pMW1047) carrying erythromycin resistant (*ereB*) gene. This vector also contains a *R.prowazekii* citrate synthase gene (*gltA*). Successful ligation of the *tlyC* into pMW1047 was confirmed by PCR, *SalI/XhoI* restriction digest and sequencing. We determined the erythromycin susceptibility of *R. typhi* and *R. akari* (MIC for *R. akari* is 0.4 ug/ml vs. *R.typhi* 10 ug/ml). Ligated recombinant plasmid was used in transformation of nonhemolytic *R. akari*. Transformants were separated under erythromycin pressure and assayed by PCR for *tlyC* gene presence. Our study demonstrates a new approach in rickettsial transformation. The presence of the *tlyC* gene into *R. akari* may confer hemolytic activity to otherwise nonhemolytic *R. akari* .

SirC AND SirA CAN INDUCE EXPRESSION OF SALMONELLA INVASION GENES INDEPENDENTLY OF HilA

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Salmonella typhimurium utilizes a specialized secretion system to deliver proteins into eukaryotic cells. This type III secretion system (TTSS) encoded within *Salmonella* pathogenicity island 1 (SPI-1) is required for bacterial entry into host cells and virulence. The genes required for the TTSS are transcriptionally regulated in response to environmental signals by a number of regulatory loci. These regulators are encoded within and without SPI-1 and include systems that have the potential to direct expression of genes in response to environmental signals. SirA is encoded outside the island, belongs to the response regulator family, and is a regulator of *sirC* expression. SirC is an AraC-like regulator encoded within SPI-1. Unlike other SPI-1 genes, expression of *sirC* does not require HilA. Overexpression of *sirC* or *sirA* restores expression of a subset of SPI-1 genes, which include *invF* and *sspC*, but not *prgH*, in the absence of HilA. This defines a HilA-independent pathway to invasion gene expression that includes SirA, SirC, and InvF. The contribution of HilD, another AraC-like regulator encoded within SPI-1, to this pathway will be discussed. The HilA-independent activation of SPI-1 gene expression may be important for the efficient assembly and function of the TTSS.

A K ANTIGEN FROM *SINORHIZOBIUM MELILOTI* THAT IS IDENTICAL TO THE SEROGROUP Y CAPSULE OF *NEISSERIA* PROMOTES AN AUXILIARY INFECTION OF THE HOST.

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Sinorhizobium meliloti is a narrow host-range microsymbiont of legumes, which is defined by the ability to elicit and infect root nodules on alfalfa (*Medicago sativa).* Many studies have shown that the infection process is actively promoted by bacterial extracellular polysaccharides (EPS) or capsular polysaccharides (K antigens), but that the two products may function is separate pathways. To study this question, we have begun to work with the "model" legume, *Medicago truncatula.* When two wild-type strains of *S. meliloti* were inoculated onto the roots of *M. truncatula* cv. A17 they exhibited distinct phenotypes: Strain NRG185 exhibited rapid (one week) nodulation of *M. truncatula* cv. Jemalong, whereas strain NRG247 required eight weeks to establish nitrogen-fixing nodules (auxiliary infection). We then tested various EPS (*exo*) and K antigen (*rkp*) mutants and found that the EPS promotes rapid infection and the K antigen promotes auxiliary infection.

The K antigen was characterized by GC-MS, FAB-MS, and NMR analyses, and shown to consist of disaccharide repeating units of glucose and sialic acid in a linkage identical to that of the serogroup Y capsule from *Neisseria meningitidis* $[\rightarrow 6)$-α-D-Glc*p*-$(1\rightarrow 4)$-α-*N*-AcetylNeu*p*-$(2\rightarrow]_n$. This is the fifth K antigen to be completely characterized from *Sinorhizobium* spp., and all conform to a conserved motif: $[\text{Hex-Kdx}]_n$, where Hex is a hexose and Kdx, is a 1-carboxy-2-keto-3-deoxy sugar. This suggests that there may be a highly conserved secondary structure, despite the differences in primary sequence, which would explain the similar biological activity in the host. Subsequent conformational analysis of the K antigen, using NMR and Molecular Dynamics (MD), yielded a helical structure with the carboxylate groups oriented inwards towards the axis of the helix. Recent MD simulations indicate that this helix may be common to the $[\text{Hex-Kdx}]_n$ motif.

A *hrcC* MUTANT OF *SINORHIZOBIUM FREDII* PRODUCES MODIFIED LPS AND NOVEL GLYCOSAMINORHAMNANS, AND LACKS K ANTIGEN.

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Sinorhizobium fredii is a broad host-range symbiont that nodulates more than eighty species of legumes, including soybean. The infection of soybean by *S. fredii* USDA257, however, is normally restricted to primitive cultivars, such as *Glycine soja* or *G. max* cv. Peking. Importantly, the introduction of a mutation into *nolW* extends the host range to the improved soybean cultivars used in North American agriculture, apparently because of the inactivation of an avirulence factor (Meinhardt, L.W., Krishnan, H.B., Balatti, P.A., and Pueppke, S.G. 1993. Mol. Microbiol. **9**:17-29). The *nolW* gene shares significant homology with *hrcC/yscC*, which belong to a family of genes in pathogenic bacteria, encoding type III secretion systems (TTSS) that function in protein secretion in pathogenesis. This report presents the first evidence that disruption of the TTSS in *S. fredii* results in significant changes in the bacterial cell wall, including a modified production of the lipopolysaccharides (LPS) and outer membrane proteins, and a lack of capsular polysaccharides (K antigens). Some of the observed changes are similar to those associated with bacterial morphogenesis to the bacteroid state, which is the endophytic (host-intracellular) form of rhizobia. We also found that cultured cells of the TTSS mutant, but not the *wt* strain, produce novel glycosaminorhamnans, which appear to be bacteroid-specific polysaccharides that are abnormally expressed by *S. fredii hrcC*. These phenomena suggest that the *S. fredii* TTSS is part of a regulatory system that governs cell morphology in response to environmental and host-derived stimuli. And why does the TTSS mutant have an extended host range? Present evidence indicates that the avirulence factor that restricts the host range of the *wt* strain may be the K antigen [→)3-β-D-Man*p*-(1→5)-β-D-Kdo*p*-(2→], which is not produced by the *hrcC* mutant.

cAMP RECEPTOR PROTEIN OF *VIBRIO VULNIFICUS:* A GLOBAL VIRULENCE REGULATOR OF ADHERENCE, EXOTOXIN PRODUCTION, CYTOTOXICITY, COLONY MORPHOTYPE, AND LETHALITY TO MICE

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Vibrio vulnificus, an estuarine bacterium, causes a fatal septicemia in susceptible subjects after ingestion of raw seafoods. Various virulence factors have been suggested to be responsible for the pathology of the disease. Regulatory mechanisms of the virulence factors are not studied well in *V. vulnificus.* In the present study, we cloned *V. vulnificus* cAMP recptor protein gene (Vv-*crp*) and investigated the global virulence regulatory role of CRP. The Vv-*crp* gene was composed of 633 nucleotides, the same size as the *V. cholerae crp.* The gene showed high homologies of 86% and 97% with that of *V. cholerae* at DNA and amino acid level, respectively. Mutation of Vv-*crp* resulted in a very pleiotrophic change of the virulence phenotypes. The mutant showed a 10-fold decrease in the adhesion to HeLa cell line. Glucose significantly inhibited adhesion of the wild type strain, which was reversed by exogenously added cAMP. The Vv-*crp* mutation decreased the production of hemolysin, the most prominent exotoxin implicated in the pathogenesis. The the hemolysin production in the wild type strain was shut by 0.2% glucose, which was also reversed by exogenous cAMP in a dose dependent manner. Cytotoxicity of the bacterium, measured by LDH release from HeLa cells, significantly decreased by the mutation. The mutation converted the colony morphotype from opaque to translucent type. The morphotype shift implied decrease in the capsule production. The decrease in the capsule production was accompanied by the increase in the cell surface hydrophobicity. These changes in the mutant were fully complemented *in trans* by a plasmid harboring the wild type gene. When the mutant was administered to CD-1 mice intraperitoneally, the LD_{50} increased by 25-fold in comparison with the isogenic wild type strain. Taken together, CRP seems to play a global regulatory role over various virulence genes of *V. vulnificus.*

EPITOPE MAPPING OF PSPA/EF3296 IN STREPTOCOCCUS PNEUMONIAE

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Several pneumococcal antigens are capable of eliciting protective response against pneumococci. Some of these proteins like pneumolysin, pneumococcal surface protein A (PspA) and pneumococcal surface adhesin A are being evaluated for their ability to induce immunity to multiple capsular types. Pneumococcal surface protein A (PspA) is a virulence factor of *Streptococcus pneumoniae* that can elicit a protective antibody response. This protein exhibits serologic and structural variability. In spite of this variability, many of the protection-eliciting epitopes of different PspAs are cross-reactive, and immunization with a single PspA can elicit protection against strains expressing different capsular polysaccharide types and serologically divergent PspAs. The *pspA* gene of strain Rx1 encodes a 65 kDa molecule composed of 588 amino acids. The N-terminal 288 amino acids are highly charged, and predict and α–helical coiled-coil protein structure. The major cross-protection eliciting residues of PspA/Rx1 have been mapped to amino acids 192-260. PspA/EF3296 is serologically distinct from PspA/Rx1. Sequencing of the molecule has revealed that the greatest amount of between the two proteins is in the α-helical portions, which are surface-exposed, and probably under selective pressure to diversify serologically. The protein products of overlapping cloned fragments of the α-helical and proline-rich domains of EF3296 *pspA* have been expressed as fusion proteins with mouse dihydrofolate reductase and are being evaluated for their ability to protect against both carriage and sepsis. Immunization with the N-terminal 115 amino acid fragment, with a larger fragment extending from 75-490 amino acids elicited protection in BALBc/ByJ mice suggesting that these region of PspA may also be important for elicitation of protective antibody.

IMMUNE SURVEILLANCE OF VIRAL INFECTIONS

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MHC class I molecules display on the cell surface oligopeptides derived from a cell's expressed genes. This allows cytotoxic T lymphocytes of the immune system to detect and eliminate cells expressing "foreign" sequences (e.g. from a viral infection or mutation). In many cases two antigen presentation pathways are involved in this process. To initiate responses, antigens must be displayed on class I molecules of professional antigen presenting cells (e.g. dendritic cells). If these cells are not themselves making the antigen, they acquire them from the extracellular fluids and present them on class I molecules via mechanisms that will be discussed. Once cytotoxic T lymphocytes are stimulated they then seek out all cells that are synthesizing the "foreign antigen" and displaying its fragments on class I molecules. The majority of these MHC class I-presented peptides are generated by large proteolytic particles, proteasomes, which are present in the cytoplasm and nucleus of all Eukaryotic cells. These peptides must be of an exact size (8-10 residues) in order to bind to class I molecules. Where examined, the proteasome is responsible for making the proper C-terminal cleavage to produce antigenic peptides, however other activities, aminopeptidases, can trim the N-terminus of peptides to the proper size. The activity of both the proteasome and aminopeptidases can be regulated by proinflammatory cytokines. After they are generated in the cytoplasm these peptides are transported into the endoplasmic reticulum where they are bound by MHC class I molecules which transport them to the cell surface for display.

IDENTIFICATION OF MURINE MACROPHAGE GENES INDUCED BY *SALMONELLA TYPHIMURIUM* INFECTION USING GENE ARRAYS

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Gene array technology offers an unprecedented opportunity to simultaneously study changes in the expression of hundreds of genes. This allows us to gain a more comprehensive understanding of how host cells respond to microbial infection. Using the well-characterized murine typhoid model as a basis for study, arrays containing nearly 600 known partial cDNAs were used to investigate altered gene expression in murine macrophages upon *Salmonella typhimurium* infection. RAW 264.7 cells were activated with interferon-γ and RNA isolated from uninfected and infected cells after four hours. Radiolabeled cDNA probes were synthesized and the relative hybridization of each probe to two identical gene arrays was quantified by phosphoimager analysis. *S. typhimurium* infection induced a greater than four-fold change in the expression of numerous genes encoding chemokines, surface receptors, cell signaling molecules, and transcriptional activators. Our results reveal differential expression of several genes which had not previously been implicated in host response to *S. typhimurium* infection. In addition, we confirm previously identified genes which validates the use of this technique. These alterations in host gene expression are currently being confirmed and quantified by Northern blots and the relative contribution of bacterial LPS signaling is being assessed in order to isolate specific *S. typhimurium*-induced changes.

ELUCIDATING THE MOLECULAR INTERACTIONS BETWEEN ß-LACTAMASE AND ß-LACTAMASE INHBITOR PROTEIN

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The rapid evolution and spread of bacteria resistant to antibiotics has become an increasing health concern. The production of the enzyme ß-lactamase by both gram-positive and gram-negative bacteria is the most common resistance mechanism to ß-lactam antibiotics, which includes the penicillins and cephalosporins. ß-Lactamase inhibitory protein (BLIP) has been shown to be a potent inhibitor of several ß-lactamases including TEM-1 ß-lactamase (K_i = 0.1 nM). The co-crystal structure of TEM-1 ß-lactamase and BLIP reveals the contact residues involved in the interface between the enzyme and inhibitor. To determine the residues in TEM-1 ß-lactamase that are critical for binding BLIP, phage display was employed. Random mutants of TEM-1 ß-lactamase in the 99-114 loop-helix and 235-240 B3 ß-strand regions were displayed as fusion proteins on the surface of M13 bacteriophage. Functional mutants were selected based on the ability to bind BLIP. After three rounds of enrichment, the sequence of a collection of functional ß-lactamase mutants revealed a consensus sequence for the binding of BLIP. Seven loop-helix residues including D101, L102, V103, S106, P107, T109, and H112 and three B3 ß-strand residues including S235, G236, and G238 were found to be critical for tight binding of BLIP. In addition, the selected ß-lactamase mutants A113L:T114R and E240K were found to increase binding of BLIP by over 6- and 11-fold, respectively. Combining these substitutions resulted in 550-fold tighter binding between the enzyme and BLIP with a K_i of 0.40 pM. Currently, randomly minimized BLIP fragments have been expressed on the surface of M13 bacteriophage. Enrichment of the minimized BLIP mutants will be performed in the presence of ß-lactamase to identify regions of BLIP that are sufficient for binding and inhibiting the enzyme. Understanding the molecular interactions between BLIP and TEM-1 ß-lactamase may aid in the development of novel inhibitors of ß-lactamase based on the structure of BLIP.

SPECIES, POPULATION AND AGE DIVERSITY IN CELL RESISTANCE TO ADHESION OF *NEISSERIA MENINGITIDIS*

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The variation of cell resistance to adhesion of meningococci serogroups A, B and C and influenza viruses was investigated in 11 animal species and in different human age groups (1st, 2nd, 3rd and 4th weeks; 2nd-3rd months; 4th-12th months, 2nd-3rd years; and 18th-60th years of life) as well as in women during pregnancy (17th-36th weeks) and childbirth. This arrangement have been chosen because resistance all of animals and of under one month-children to this disease in contrast, for instance, to influenza infection at the same age. 257 children up to four years of age and 5137 adults were tested. All of the adhesins preparations used have been stabilized by lyophilization to provide good test standardization. It was shown that red blood cells of all animals tested (17 goats, 17 sheep, 17 donkeys, 8 mice, 8 guinea pigs, 5 rats, 1 Syrian hamsters, 24 rabbits, 5 horses, 3 cows, 14 hens) as well as of 103 human newborns were absolutely resistant to attachment of meningococci. In neonatal and followed periods the human cells become far different individually sensitive to meningococcal adhesion. In contrast, the viral adhesion was characterized by different individual cell sensitivity in all species and all age groups tested. Pregnancy and birth time did not influence on the women's cell sensitivity to adhesion of *N.meningitidis*. Different receptors are involved into interactions of human cells with influenza viruses and meningococci. The function of meningococcal receptors on human cells develops during postnatal ontogenesis. A positive correlation between adhesive potencies of erythrocytes, leukocytes and epitheliocytes was revealed. The differences observed can repulse specific, individual and ontogenetic variations in natural immunity to meningococcal infection. Innate immunity of a whole organism is determined by innate immunity of its cells. It can be revealed ***in vitro***.

BACTERIAL MODULATION OF PULMONARY NEUTROPHIL INFLUX. Russo TA, Davidson BA, Carlino UB, Helinski J, Knight PA III
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Enteric Gram-negative bacilli (GNB) are capable of causing life-threatening pneumonia. Neutrophils are critical in protecting against infection in this site. Prior to considering manipulating the host biologic response as a treatment modality in Gram-negative pneumonitis, it is critical to understand the mechanisms by which the host responds to bacterial challenge and how certain bacterial components modulate this response. This will enable one to achieve maximal bacterial clearance while minimizing host-factor mediated pulmonary damage. Outside of studies on lipid A, little information is available on other Gram-negative factors that may affect pulmonary neutrophil influx.

We hypothesized that capsular polysaccharide (K54) and O-specific antigen (O4), surface components present in most GNB, may modulate neutrophil influx into the lungs. To test this hypothesis a wild-type, extraintestinal *E. coli* strain (CP9, O4/K54/H5) and three isogenic derivatives deficient in: a) K54, b) O4), or c) both were used as challenge strains in a rat model of acute pulmonary infection. Myeloperoxidase activity (MPO) was measured to assess for neutrophil influx.

MPO was measured for a given strain at several different challenge inocula (CI) at 0, 1, 3, 6, 9, 12, and 15 hours post-challenge. After bacterial challenge, MPO increased, reaching a plateau level 6-8 hours post-challenge. Maximal MPO increased approximately 10-25-fold depending on the challenge strain and CI utilized. Analysis demonstrated that MPO depended primarily on the initial CI. To determine the effects of K54 and O4 on MPO, a logistic function model was used to describe the MPO as a function of time for each of the strains and CI.

In the absence of the K54 capsule 8.99 times the CI is necessary to achieve the same maximum MPO, relative to a K54 positive strains ($p < 0.0001$). The absence of the O4 antigen had a diametric effect on neutrophil influx compared to the loss of K54. In the absence of O4 0.36 times the CI is necessary to achieve the same maximum MPO, relative to a O4 positive strains ($p = 0.0032$). No interactive effects were observed between K54 and O4. These observations support the hypothesis that the K54 capsule is a proinflammatory mediator that stimulates the host defense response and that the O4 antigen attenuates this response. Mechanistic studies are in progress which may enable us to modulate this critical host defense response and thereby improve our treatment of Gram-negative pneumonitis.

RELEASE OF HISTAMINE FROM HUMAN PERIPHERAL BLOOD LEUKOCYTES, CULTURED MAST CELLS AND BASOPHILS BY STREPTOCOCCAL PYROGENIC EXOTOXIN (**SPE-B**)/ STREPTOCOCCAL CYSTEINE PROTEINASE (**SCP**)

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Streptococcus pyogenes causes serious diseases in human, such as streptococcal toxic shock syndrome (STSS). A number of exotoxins such as SPE-A, SPE-B/SCP, and SPE-C has been implicated as possile causative agents of STSS. In this study, we examined whether or not SPE-B/SCP is able to release histamine from mast cells and basophils. SPE-A wasa used as control.

We generated mast cells by culturing human cord blood mononuclear cells for 12 to 18 weeks in the presence of rSCF and rIL-6, and basophils from the same source by culturing in the presence of rIL-3 for 5 to 6 weeks. Human peripheral leukocytes containing basophils were also used in these experiments. Mast cell and basophils degranulation was determined morphologically by May-Gr¸newald Giemsa stain and electron microscopy. Histamine production was analyzed in triplicate by HPLC.

Mast cells (1 to 5 x 10^4), basophils (1 to 5 x 10^5) and peripheral leukocytes (1.5 to 2.5 x 10^6) in 500 µl of Tyrode's solution (pH 7.4), respectively, were incubated with 5 to 20 µg/ml of SCP or SPE-A for 30 or 60 min at 37°C. All the above cells released histamine by stimulation with SPE-B/SCP in a dose dependent fashion, and the degranulation of the cells was observed morphologically. Alpha-lactic dehydrogenase (LDH) was not released from the cells. The SPE-B/SCP lost the histamine releasing capacity by heating at 60°C for 30 min, and this capacity was also inhibited by the proteinase inhibitor, E64. SPE-A did not have any effect on the release of histamine. Induction of histamine release by SPE-B /SCP requires influx of calcium into the cells. Histamine release was completely blocked by 5 mM EGTA. Calcium influx was unusually delayed compared with that of IgE-mediated histamine release, subsequent to which histamine release could be observed. Taken together, SPE-B/SCP may interact with its substrate(s) on the cell surface and trigger an initial event of degranulation response.

Histamine release induced by SPE-B/SCP may play a role in the STSS and streptococcal infection of skin and mucous membrane.

Salmonella typhi Ty2 LACKS THE CHROMOSOMAL REGION ENCODING THE ADJACENT *ompD* AND *smvA* GENES PRESENT IN THE *Salmonella typhimurium* GENOME.

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Salmonella typhi causes a systemic disease only in its human host. The closely related *Salmonella typhimurium* causes a typhoid-like systemic disease in the mouse, but causes only a self-limited gastroenteritis in the human host. Although these two species share about 98-99% DNA sequence similarity, they display a remarkably different host specifity. This contrasting ability to infect their respective hosts may lay in profound genome differences

In this study we have shown that the majority of *Salmonella* species most frequently associated with the systemic infection of vertebrate hosts produce a major outer membrane porin OmpD. However, this protein is absent from the outer membrane protein profiles of *S. typhi* strain Ty2 and 14 clinical isolates examined by SDS-PAGE. PCR primers flanking the *ompD* coding sequence amplify this gene from the genomes of all tested *Salmonella* species, with the exception of *S. typhi* strains. Consistent with this result, a PCR-amplified *ompD* fragment do not hybridize with DNA isolated from the *S. typhi* strains.

We have extended this study to *smvA* gene, which is mapping next to *ompD* in the *S. typhimurium* chromosome and is responsible for the methyl viologen resistance phenotype. In contrast to all *Salmonella* species tested, *S. typhi* strains presented a methyl viologen sensitive phenotype. As suggested by hybridization and PCR amplification analyses, *S. typhi* Ty2 chromosome is also missing the *smvA* gene, however *smvA* is present in the clinical isolates tested. We have introduced *S. typhimurium ompD* and *smvA* genes into *S. typhi* Ty2 by generalized transduction with phage P22. The recombinant *S. typhi* Ty2 strain harboring *ompD* and *smvA* genes expresses OmpD in the outer membrane, however remains methyl viologen sensitive. Taken together *ompD* deletion and methyl viologen sensitive phenotype may contribute to the highly restricted host-specificity of *S. typhi*.

CHARACTERIZATION OF PLS, THE ADHESION REGULATING SURFACE PROTEIN OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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Pls is a 230kDa surface protein expressed by a group of methicillin resistant *Staphylococcus aureus* (MRSA) strains with poor adherence ability to several plasma and extracellular matrix proteins such as immunoglobulin G and fibronectin. Pls has typical features of the staphylococcal surface proteins with its signal sequence, the cell wall anchoring sequence LPDTG and the positively charged C-terminus. The protein has three areas of extensive repeats, one of which is a serine-aspartate region also present in a few other *S. aureus* proteins and the two others not homologous to known proteins. The repeat regions vary in size between different MRSA strains, causing size variation of Pls. In a pulsed field gel electrophoresis analysis of *S. aureus* total DNA a *pls* probe hybridizes with the same SmaI fragment as the methicillin resistance encoding *mecA* gene probe suggesting a possible common origin of the genes in one evolutional lineage of MRSA.

We have constructed a chromosomal site-specific *pls* knockout mutant, whose protein A and fibronectin binding protein mediated adherence is much increased. Pls is also cleaved as a result of blood plasminogen activation into plasmin on the bacterial surface *in vitro*, leading to an improved adherence. Plasmin is a strictly controlled serine protease that several pathogens have been shown to utilize in invasion and penetration of the extracellular matrix. We think that Pls plays a role in regulation of bacterial adhesion.

TWO ARAC/XYLS FAMILY MEMBERS CAN INDEPENDENTLY COUNTERACT THE EFFECT OF REPRESSING SEQUENCES UPSTREAM OF THE *HILA* PROMOTER

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During infection of its hosts, *Salmonella typhimurium* enters the epithelial cells of the small intestine. This process requires a number of invasion genes encoded on *Salmonella* pathogenicity island 1 (SPI1), a 40 kb stretch of DNA located near minute 63 of the *S. typhimurium* chromosome. Expression of *S. typhimurium* SPI1 invasion genes is activated by the transcription factor HilA. *hilA* is tightly regulated in response to many environmental conditions, including oxygen, osmolarity, and pH. Regulation of *hilA* expression may serve to limit invasion gene expression to the appropriate times during *Salmonella* infection. We have mapped the transcription start site of *hilA*, and identified regions of the promoter that are required for repression of *hilA* expression by conditions unfavorable for *Salmonella* invasion. We have also identified two SPI1 encoded genes, *hilC* and *hilD*, that can independently derepress *hilA* expression. HilC and HilD are both members of the AraC/XylS family of transcriptional regulators. *Salmonella* may encode two derepressors of *hilA* expression to differentially regulate invasion genes in diverse hosts or tissues. Our results indicate that *hilD* is more important than *hilC* for invasion of cultured cells after bacterial growth in rich media. We are currently examining the effects of *hilC* and *hilD* mutations on *S. typhimurium* infection of mice.

BACTERIAL PRODUCT STIMULATION OF MACROPHAGES IS INHIBITED BY CATIONIC ANTIMICROBIAL PEPTIDES

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Macrophages produce numerous inflammatory mediators upon stimulation by bacterial products. Stimulation of macrophages by LPS but not other bacterial products such as lipoteichoic acid (LTA) and bacterial DNA (CpG DNA) is enhanced by the presence of lipopolysaccharide binding protein (LBP). In fact, in the absence of LBP, LTA-stimulation of macrophages is enhanced. Cationic peptides, which are prevalent throughout nature as part of the intrinsic defenses of most organisms, have broad spectrum antimicrobial activity. They are also able to synergize with other antimicrobial agents in killing resistant bacteria. Since they are antimicrobial and some have been shown to bind LPS, it was of interest to determine if they would protect cells from the effects of LPS and other bacterial products. In this study, a series of structurally varied peptides were tested for their ability to bind LPS and LTA as well as block production of cytokines by macrophages stimulated with various bacterial products. They were shown to have a high affinity for different species of LPS as well as LTA. They were also shown to inhibit macrophage derived TNF and IL-6 in response to LPS, LTA and to some degree CpG DNA and boiled *S. aureus*. In the case of LPS, it was hypothesized that the peptides acted by binding LPS and preventing interaction with LBP and host cells. Indeed, peptides do block the LBP-LPS interaction as tested by an LBP ELISA-like assay and that inhibition of LPS-LBP interaction correlated with inhibition of cytokine production by macrophages stimulated with LPS. There is evidence that the peptides also have other effects as they can inhibit TNF production by macrophages even when added up to 60 minutes after LPS. Thus these cationic antimicrobial peptides have a broad range of effects including affinity for LPS and LTA as well as inhibition of bacterial product stimulation of macrophages.

ACTIVATION OF HUMAN MONOCYTIC CELLS BY *BORRELIA BURGDORFERI* AND *TREPONEMA PALLIDUM* IS FACILITATED BY CD14 AND CORRELATES WITH SURFACE-EXPOSURE OF SPIROCHETAL LIPOPROTEINS

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We examined the involvement of CD14 in monocyte activation by the etiologic agents of Lyme disease (*Borrelia burgdorferi*) and syphilis (*Treponema pallidum*). *B. burgdorferi* induced secretion of IL-8 by vitamin D_3-matured THP-1 cells which was inhibited by a CD14-specific mAb known to block cellular activation by LPS and the prototypic spirochetal lipoprotein, outer surface protein A (OspA). Enhanced responsiveness to *B. burgdorferi* also was observed when THP-1 cells were transfected with CD14. Because borreliae within the mammalian host and *in vitro*-cultivated organisms express different lipoproteins, experiments also were performed with "host-adapted" spirochetes grown within dialysis membrane chambers implanted into the peritoneal cavities of rabbits. Stimulation of THP-1 cells by host-adapted organisms was CD14-dependent and, interestingly, was actually greater than that observed with *in vitro*-cultivated organisms grown at either 34°C or following temperature-shift from 23°C to 37°C. Consistent with previous findings that transfection of Chinese hamster ovary (CHO) cells with CD14 confers responsiveness to LPS but not to OspA, *B. burgdorferi* failed to stimulate CD14-transfected CHO cells. *T. pallidum* also activated THP-1 cells in a CD14-dependent manner, although its stimulatory capacity was markedly less than that of *B. burgdorferi*. Moreover, cell activation by motile *T. pallidum* was considerably less than that induced by treponemal sonicates. Taken together, these findings support the notion that lipoproteins are the principle component of intact spirochetes responsible for monocyte activation, and they indicate that surface exposure of lipoproteins is an important determinant of a spirochetal pathogen's proinflammatory capacity.

INVASION PLASMID ANTIGEN-C (IpaC) OF *SHIGELLA FLEXNERI* BINDS DIRECTLY WITH β-CATENIN WITHIN THE HOST CELL DURING THE PROCESS OF INFECTION

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Shigella, the most prevalent etiological agent of bacillary dysentery, invades the epithelial cells of colon, multiply within and spread into the neighboring cells. Ipa (*i*nvasion *p*lasmid *a*ntigen) proteins – particularly IpaB and –C play crucial role during the disease process. Both the proteins synthesized and secreted into the cytoplasmic milieu once the bacterium make its entry within host cells and modulate the host cellular function. Though IpaB has been shown to bind interleukin-1β converting enzyme (ICE) and leads to the cellular apoptosis, no such host cell molecule has yet been identified which binds IpaC. In search of the binding partner of IpaC we employed yeast two-hybrid system using IpaC as bait and screening of HeLa cDNA library leads to the identification of β-catenin, a vertebrate homologue of *Drosophila* armadillo protein implicated in cell adhesion and Wnt signaling. In vivo and in vitro experiments confirmed this interaction by co-immunoprecipitation and gel-overlay assay, respectively. Furthermore, these two proteins can be colocalized in the cytoplasm of the infected host cell. It was also observed that β-catenin got tyrosine phosphorylated upon infection. The binding site of IpaC has been identified within the ninth armadillo repeats of β-catenin. These results may suggest that IpaC can modulate the post invasion scenario by binding directly with β-catenin affecting its interaction with E-cadherin – molecule necessary for cell to cell spread of shigellae.

REGULATION OF INTRACELLULAR BACTERIAL GENE EXPRESSION IN *L. MONOCYTOGENES*

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Listeria monocytogenes is a gram positive facultative intracellular parasite that is responsible for serious disease in immunocompromised patients and pregnant women. The bacteria invade host cells, escape from the vacuole, and spread to adjacent cells using an actin based motility mechanism. *L. monocytogenes* is capable of sensing the different host cell compartment environments it encounters during the course of infection and responding with the regulated expression of bacterial virulence genes. The expression of several virulence determinants is dependent upon a transcriptional activator known as PrfA. We have examined PrfA-dependent activation of *actA*, a gene whose product is required for intracellular bacterial motility, under a variety of environmental conditions and within host cells using a B-glucoronidase (GUS) reporter system. GUS provides a sensitive means of quantitatively comparing intracellular and extracellular levels of *L. monocytogenes* gene expression. Expression of *actA* was found to increase over 500-fold for cytosolic *L. monocytogenes* when compared to bacteria grown in BHI broth. *actA-gus* transcriptional fusions were additionally used to compare the effects of PrfA DNA binding site substitutions on *actA* expression levels. Substitution of a high affinity PrfA DNA binding site for the low affinity site found in the *actA* promoter produced no significant effect on *actA* expression, indicating that an increase in PrfA binding to the *actA* promoter did not result in higher levels of *actA* expression. Introduction of a PrfA(Gly145Ser) mutant allele that results in the hypersecretion of ActA as well as several other *L. monocytogenes* virulence factors increased *actA-gus* expression in broth grown cultures, but levels were still significantly lower than those observed for intracellular bacteria. No significant difference was detected with respect to the ability of PrfA (Gly145Ser) to activate expression from the high versus low PrfA-affinity promoters in the intracellular environment. Our data indicate that neither substitution of a high affinity PrfA DNA binding site nor the introduction of an ëactivatedí PrfA allele is sufficient to increase *actA* expression to the levels observed for intracellular bacteria. Optimal expression of *actA* may require additional factors or signals that are generated once *L. monocytogenes* reaches the host cell cytosol.

FUNCTIONAL AND GENETIC ANALYSIS OF H19B OPERATOR-REPRESSOR INTERACTIONS

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In many EHEC strains the genes encoding Shiga toxin (Stx) are carried by prophages. Two of these phages, H-19B and 933W, are members of the λ family. We have proposed that production and release of Stx from these EHEC strains is largely controlled by phage encoded regulatory functions. Phage repressor binding to operator sequences ultimately controls the expression of these regulatory functions. Since prophage induction would lead to phage production and bacterial death, Stx production without destruction of the entire EHEC population would require that only a subpopulation of the lysogens be induced in the gut of infected individuals, raising the question of the nature of repressor-operator interaction in *stx*-carrying phages.

Sequence comparison with other lambdoid phages identified putative operator regions and repressor gene of H-19B. Footprinting of this operator region DNA with purified repressor and results of sequencing of the genome of an H-19B mutant that grows in the presence of H-19B repressor (virulent mutant) confirmed that this initial determination was correct. The operator region of H-19B resembles that of other lambdoid phages, having operator regions flanking the cI repressor gene and binding sites that, with one exception have a partially conserved 17 bp region of two-fold rotational symmetric sequences. The right operator, O_R, like that of other lambdoid phages has three of these binding sites. The left operator, O_L, unlike other lambdoid phages, has only two binding sites and OL2 lacks the typical two-fold rotational symmetric sequences. The virulent mutant of H19B has mutations in OR1 and OL1 differing from λ virulent mutants that have, in addition to mutations in OL1, mutations in both OR1 and OR2. Possible functional consequences of these differences will be discussed.

A GENETIC ANALYSIS OF *H. CAPSULATUM* PATHOGENESIS.

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Our goal is to identify fungal genes that are required for the pathogenesis of *Histoplasma capsulatum*. This dimorphic fungus grows in hyphal or conidial forms in the soil; hyphal fragments or microconidia are inhaled by the host. At 37°C, the cells undergo a morphogenetic switch and grow as a budding yeast form which parasitizes the phagolysosome of macrophages. How *H. capsulatum* is able to escape killing by macrophages and colonize the phagolysosome, an intracellular niche that is normally hostile to microbes, is a mystery. The recent development of molecular genetic tools now makes it possible to use genetics and molecular biology to dissect the mechanism of interaction between *H. capsulatum* and the host cell.

We are using molecular genetic methodology to identify *H. capsulatum* genes that are necessary for the organism to parasitize macrophages. We are employing a previously developed cell culture assay in which *H. capsulatum* cells are co-cultured with a monolayer of mouse macrophages (Eissenberg et al., *Infect Immun*, 1991. **59**(5)). *H. capsulatum* is quickly internalized by the macrophages and lyses the macrophage monolayer after 3-5 days. EMS mutagenesis is being used to generate a bank of mutant yeasts; we will then use the monolayer lysis assay to identify mutant strains that fail to lyse macrophages. Microscopic assays will be used to sort the mutants into the following classes: those that affect adherence of the fungus to macrophages, entry into macrophages, evasion of killing by macrophages, replication within macrophages, and lysis of macrophages. Genes that are defective in the mutant strains will be cloned by complementation of the lysis phenotype in the monolayer assay. The gene products so identified will be analyzed using sequence comparison, expression pattern, and sub-cellular localization. We believe that molecular genetics, which has been key to guiding our understanding of biology in other organisms, will shed light on the pathogenesis of this fascinating fungus.

"CFTR –" CELLS ARE MORE SUSCEPTIBLE TO *S. AUREUS* INVASION BUT PERSISTENCE IS NOT DIFFERENT FROM "CFTR +" CELLS

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Cystic fibrosis is characterized by chronic pulmonary infections that eventually lead to tissue destruction. Two pathogenic organisms are predominantly found in the lung: early *S. aureus*, and *P. aeruginosa* in the later course of the disease. Several mechanisms are thought to contribute to the decreased resistance to pulmonary infections: i) a high salt content of the mucus blocks the action of antimicrobial peptides (e.g. defensins), ii) a reduced clearance of the highly viscous secretions leads to conditions favorable for bacterial growth, and iii) the lack of additional functions of CFTR (cystic fibrosis transmembrane conductance regulator), apart from its role as a Cl^- channel. *In vitro*, CFTR is used as an invasion receptor by *P. aeruginosa* (Pier G.B. *et al.*, Science 1996; 271:64). Stable expression of the intact CFTR in the mutant cell line strongly increases bacterial internalization. CFTR serves as a clearance receptor for *P. aeruginosa* in the lung, when bacteria bind to shed cells and are subsequently expelled (Pier G.B. *et al.*, PNAS 1997; 94:12088).

We found a remarkable difference for *S. aureus* using the same cell lines. In a lysostaphin protection assay for intracellular persistence, equal numbers of *S. aureus* (clinical strain) were recovered from mutant cells ("CFTR –") and CFTR-WT-complemented cells ("CFTR +") 24 h post infection. No difference in persistence between cell lines was found over time, but as expected, SCV (small colony variants) survived 1 to 1.5 log(10)-fold better than the normal strain. We have developed a flow cytometric invasion assay and have shown previously a FnBP-dependent zipper type invasion mechanism for *S. aureus* (Sinha B. *et al.*, submitted). This method allows the quantitative investigation of invasion independently of bacterial growth. We observed a 3- to 10-fold increase of invasion in "CFTR –" over "CFTR +" cells. The differences may at least partially be due to a reduced uptake in "CFTR +" cells, suggesting a direct or indirect inhibition of invasion by CFTR. Cellular invasion and intracellular formation of SCV is thought to serve as persistence mechanism for *S. aureus*. This may be one pathogenic factor for the establishment of *S. aureus* lung infection of patients with cystic fibrosis.

LON AND CLP FAMILY PROTEASES AND CHAPERONES SHARE HOMOLOGOUS SUBSTRATE-RECOGNITION DOMAINS

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Members of the Clp/Hsp100 family of chaperones and protease regulatory subunits play roles in protein quality control, tolerance to stress, control of gene expression, the inheritance of prion-like factors, and bacterial virulence. How these proteins select the proper cellular targets is a central question in understanding the function of Clp/Hsp100 family members. To better understand their substrate specificity, we have identified and characterized substrate-binding domains from members of the Clp family and found a homologous region in Lon protease. Circular dichroism, tryptophan fluorescence and limited protease digestion reveal that the domains from Lon, ClpA and ClpY are stably and independently folded. The corresponding regions from ClpB and ClpX are unstable. All five fragments exhibit distinct patterns of binding to three proteins that are substrates *in vivo*: the heat shock transcription factor σ^{32}, the SOS mutagenesis protein UmuD, and Arc repressor bearing the SsrA degradation tag. Recognition of UmuD is mediated through peptide sequences within the N-terminal region, while recognition of both σ^{32} and SsrA-tagged Arc requires sequences at the C-terminus. These results indicate that the Lon and Clp proteins use similar mechanisms of substrate discrimination and suggest that these related ATP-dependent bacterial proteases search through accessible or disordered regions of potential substrates for the presence of specific targeting sequences.

DEATH BY LETHAL INJECTION: STRUCTURE OF A NOVEL PHOSPHOTYROSINE INTERACTION DOMAIN. Craig L. Smith, James B. Bliska*, Lee G. Montagna*, and Mark A. Saper, Biophysics Research Division and Department of Biological Chemistry, University of Michigan, Ann Arbor MI 48109-1055, U.S.A., *Department of Molecular Genetics and Microbiology, SUNY Stony Brook, Stony Brook, NY 11794, U.S.A.

YopH is a modular protein that is injected into host macrophages by the contact dependent type III secretion system of *Yersiniae*. YopH inhibits phagocytosis through dephosphorylation of host proteins $p130^{Cas}$, paxillin, and focal adhesion kinase (FAK) by its carboxy-terminal phosphotyrosine phosphatase domain. Experimental evidence suggests that motifs responsible for the secretion and injection of YopH are located in the first 71 residues of the non-catalytic amino-terminal domain. Not only is the amino-terminal domain responsible for injection, as shown by Cornelis and colleagues, but it is also responsible for targeting the phosphatase domain to focal adhesions by binding to $p130^{Cas}$ and paxillin in a phosphotyrosine dependent manner. Interestingly, this domain lacks significant sequence homology to other phosphotyrosine-binding domains like SH2 and PTB and represents a new protein-protein interaction module.

We report the structure of the amino-terminal domain of YopH (YopH-NT residues 1-130) to 2.2 Å resolution. The crystal structure reveals a unique fold that is unlike that of the SH2 and PTB phosphotyrosine-binding domains. A striking feature of this domain is a positively charged region that may be the site of $p130^{Cas}$–YopH-NT interaction. Substitutions of amino acid residues in this region have been shown to inhibit the phosphotyrosine interaction but not secretion or injection.

IDENTIFICATION OF DNA UNIQUE TO THE INVASIVE BRAZILIAN PURPURIC FEVER CLONE OF *H. INFLUENZAE* BIOGROUP AEGYTIUS

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Brazilian purpuric fever (BPF) is a fatal disease that causes disseminated purpura and vascular collapse in children. The vascular destruction is a distinctive trait caused by a unique invasive clone of *Haemophilus influenzae* biogroup aegyptius (*H. aegyptius*), a bacterial species that is normally noninvasive and causes mild cases of conjunctivitis. The unusual invasive attributes of the BPF clone of *H. aegyptius* make this pathogen an ideal model to study the emergence of an invasive derivative, which expresses new virulence determinants, from an old noninvasive bacterial pathogen. In addition, little is known about the molecular and genetic nature of the BPF virulence factors and the pathogenesis of the purpura fulminans caused by this pathogen.

We have used PCR-based subtraction genome hybridization to investigate the genetic nature of the unusual invasive attributes of this pathogen. A subtracted library enriched in genomic sequences present in the BPF-causing prototype strain F3031 but absent in the noninvasive isolate F1947 was constructed. After colony blot hybridization, two subtracted clones that hybridized only with F3031 DNA, pMU33 and pMU34, were selected and evaluated in more detail. Southern analysis showed that DNA from BPF strains isolated from Brazilian patients hybridized with these two subtracted clones. In contrast, no hybridization was detected with DNA isolated from the *H. aegyptius* strain F4380, which was isolated from an Australian patient that showed the typical BPF symptoms, as well as DNA from *H. influenzae* strains belonging to type a-f. Sequence analysis of the F3031 DNA harbored by pMU33 and pMU34 revealed that they represent two different loci and no known homologs exist in the GenBank database.

These results show that unique BPF DNA sequences can be detected and isolated by subtraction genome hybridization. Ultimately, this approach will furnish new insights into the genetic diversity and evolution of this pathogen, and will provide information regarding the virulence factors of BPF-causing strains of *H. aegyptius*.

SHEDDING LIGHT ON IMMUNITY TO MYCOBACTERIA

Snewin V.A., Gares M-P., Turner D.J. and Young D.B.

Protective immunity to mycobacterial infection is incompletely understood but probably involves the co-ordinated interaction of multiple cell types and cytokines. With the aim of developing assays that might provide a surrogate measure of protective immunity, we have employed recombinant mycobacteria carrying a luciferase reporter enzyme (plasmid pSMT1), to assess the effectiveness of anti-mycobacterial immunity in model systems. Measurement of luminescence was shown to provide a rapid and simple alternative to counting of colony forming units as a means of monitoring mycobacterial viability. We describe optimisation of a luciferase reporter strain of *Mycobacterium tuberculosis* and demonstrate its application for study of mycobacterial interactions in a murine model of infection.

Having demonstrated reduction in luminescence in organs of BCG-vaccinated mice challenged with *M. tuberculosis*/pSMT1, we have developed an "ex-vivo" model to assess vaccine-induced protection. Initially spleens were removed from naive and immunised mice, homogenised to prepare a single cell suspension and infected with *M. bovis* BCG/pSMT1. Under these conditions, the challenge organism grew equally well in control and immune cultures. However, in experiments in which mice were challenged with the reporter strain *in vivo*, specific anti-mycobacterial activity was reproduced *in vitro*. Splenocytes from vaccinated animals taken even 24 hours post challenge were able to demonstrate control of mycobacterial growth compared to those from naive animals. We have established this model as a way of dissecting the complex interactions involved protective immunity to mycobacteria and to establish correlates of protection required to characterise novel vaccines.

Assessment of Immunity to Mycobacterial Infection using Luciferase Reporter Constructs. Snewin, V. A., Gares, M-P., Ó Gaora, P., Hasan, Z., Brown, I. and Young D.B. INFECTION AND IMMUNITY, in press.

THE CAPSULE PATHOGENICITY ISLAND OF *N. MENINGITIDIS*.

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The 24 kb chromosomal pathogenicity island of *Neisseria meningitidis* contains the genes necessary for the expression of capsular polysaccharide that distinguishes the meningococcus from other *Neisseria* spp. Two divergent operons responsible for capsule transport and biosynthesis (G+C content 40.3% vs. meningococcal genome 51%) are located in the island. Exchangeable biosynthesis gene cassettes determine capsule serogroup specificity (e.g., A, B, C, Y, W-135) and allow capsule switching. The island is bound on the 5N-end by an IS*1016*. The island contains genes required for capsule lipidation, *lipA* and *lipB*, and a homologue of *tex* (59.4% G+C), a novel transcriptional accessory protein important in the regulation of pertussis and adenylate cyclase toxins. The island also contains *galE* and homologues of the *E. coli* rhamnose biosynthesis genes *rfbB, A,* and *C,* partial or complete duplication of these genes (*galE*^{I}, *rfbB*^{I}, *rfbA*^{I}, *rfbC*^{I}), and homologues of HphI (C) and HphI (A) methylases of *H. parahemolyticus* (G+C content 37.9%). In *N. gonorrhoeae*, *tex* and the HphI restriction modification system homologues are present but not the capsule biosynthesis, transport or lipidation gene clusters.

The meningococcal capsule pathogenicity island has evolved from a primordial neisserial ancestor by multiple steps involving recombination of foreign DNA in the genomic region surrounding *galE*.

MOLECULAR ANALYSIS OF SIALIDASE-ENCODING GENES IN *CLOSTRIDIUM PERFRINGENS*

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The anaerobic pathogen *Clostridium perfringens* causes gangrene and a wide variety of intestinal diseases in humans and animals. The bacterium secretes many toxins as part of the disease process. One of these toxins is sialidase (or neuraminidase), which hydrolyzes sialic acid from sialoglycoconjugates. The sialidases provide nutrients to the cell in the form of sialic acid and also can disrupt normal host cell functions, many of which are dependent on sialic acid residues on the cell surface. *C. perfringens* is unusual in that it produces two sialidases, a larger secreted enzyme (NanI), and a smaller cytoplasmic sialidase (NanH). It has been proposed that NanI provides free sialic acid to the cell from complex sialoglycoconjugates and that NanH, being intracellular, cleaves sialic acid from short oligosaccharides that have been be transported into the cell. Our work and others have shown that both sialidases are induced when sialic acid is present in the growth medium. Using Southern blot analyses, we have found that a group of strains identified as food poisoning isolates, isolated in Great Britain, lack the *nanI* gene, and exhibited no detectable extracellular sialidase activity. Another isolate, strain 13, identified as a cause of gas gangrene in humans, has the *nanI* gene but lacks a copy of the *nanH* gene on the chromosome. This strain showed a low level of intracellular sialidase activity. This strain is unique, since it's been previously reported that all 2,659 isolates of *C. perfringens* tested had the *nanH* gene. We have cloned the *nanI* gene from strain 13 and have identified three sialic acid-inducible promoters by primer extension. A derivative of a *nanI- C. perfringens* isolate (strain SM101) was transformed with a plasmid carrying the *nanI* gene and its promoters, but it was not expressed in these cells. In contrast, the *nanH* gene, from strain SM101, after transformation into the *nanH-* strain 13, was expressed in this host. These results indicate that both the toxin gene repertoire and regulation of the sialidase-encoding genes differ between the two strains.

HELICOBACTER PYLORI IN THE STOMACH AND DUODENUM OF PATIENTS WITH DUODENAL ULCER AND ASYMPTOMATIC SUBJECTS

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Background: Approximately half of the world's population is infected by *Helicobacter pylori*. The organism colonizes the epithelium of the stomach and areas of gastric metaplasia in the duodenum. *H. pylori* infection may result in chronic active gastritis, peptic ulcer disease, and most probably gastric cancer but only 10-15% of those infected develop significant symptoms, e.g. duodenal ulcer. The aims of these studies were to compare the extent of colonization of *H. pylori* in the antrum of the stomach and the duodenum of duodenal ulcer (DU) patients and asymptomatic subjects (AS). It was also studied if phenotypic properties or genotypic markers of *H. pylori* strains may be related to the development of duodenal ulcer. **Materials & Methods:** From 21 DU patients and 20 AS, 1-2 antral biopsies and 4-5 duodenal biopsies were collected and cultured for *H. pylori* colony forming units (CFU). The active and chronic inflammatory score were analyzed in duodenal biopsies by histology. The strains isolated were analyzed for the cytotoxin associated (*cagA*) gene by PCR and for production of vacuolating cytotoxin A (VacA) using a modified HEp-2 cell test. The *H. pylori* blood group antigen-binding adhesin (BabA) was studied using a Lewis b oligosaccharide probe and specific antigens were determined both qualitatively and quantitatively by direct and inhibition ELISAs using monoclonal antibodies. DNA patterns of *H. pylori* isolates were analyzed by PCR using different random primers and ribotype patterns were confirmed by Southern Blotting. **Results:** Similar number of *H. pylori* were colonizing antral biopsies from DU patients and AS. In the duodenum, however, the density of *H. pylori* bacteria, the number of neutrophils, the extent of gastric metaplasia, and colonization of type I (*cagA*$^+$, VacA$^+$) strains were significantly higher in DU patients as compared to in AS. The *cagA*, the blood group antigen Lewis y and BabA were found in significantly higher frequencies in *H. pylori* strains isolated in the duodenum from DU patients than in corresponding strains from AS. *N*-acetyl-neuroaminyllactose-binding-hemagglutinin, flagellins, urease, a 26 kDa protein and a neutrophil-activating-protein were found on almost all duodenal and antral *H. pylori* strains isolated from DU and AS. Genotypic differences were observed in *H. pylori* strains isolated from the duodenum as compared to from the antrum in DU patients. Different DNA profiles among duodenal strains isolated from the same DU patient were also found. **Conclusions:** The number and characteristics of *H. pylori* were comparable in the antrum of DU patients and AS, whereas considerably higher densities of bacteria were found in the duodenum od DU patients and putative virulence markers, e.g. *cagA*, BabA and Ley antigen were more predominant in duodenal strains from DU patients than from AS. Genotypically as well as phenotypically different *H. pylori* strains were isolated from the antrum as compared to from the duodenum in many of the DU patients, suggesting that a mixture of different *H. pylori* strains may colonize the same patient. These studies indicate the importance of studying *H. pylori* infection in the duodenum, in an attempt to identify strains and virulence factors that may contribute to the development of duodenal ulcer.

COMPLETE DNA SEQUENCE AND STRUCTURAL ANALYSIS OF THE EPEC ADHERENCE FACTOR (EAF) PLASMID OF ENTEROPATHOGENIC *ESCHERICHIA COLI*

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The complete nucleotide sequence and organization of the EAF plasmid of enteropathogenic *E. coli* (EPEC), strain B171 (O111:NM), were determined. The EAF plasmid encodes two known virulence-related operons, the *bfp* operon which composed of genes necessary for bundle-forming pili (BFP) biosynthesis, and the *bfpTVW* (*perABC*) operon, composed of regulatory genes required for *bfp* transcription and also for transcriptional activation of the *eae* gene in the LEE pathogenicity island on the EPEC chromosome. Besides the *bfp* and *bfpTVW* (*perABC*) operons, the 69 kb EAF plasmid, henceforth designated pB171, contains potential virulence-associated genes, plasmid replication and maintenance genes, and many insertion sequence elements. Of the newly identified ORFs, two ORFs which comprise a single operon were found that would encode proteins with high similarity to a C-terminal region of ToxB whose coding sequence is located on pO157, a large plasmid harbored by enterohemorrhagic *E. coli*. Another ORF, located between the *bfp* and *bfpTVW* operons, showed high similarity with *trcA*, a *bfpT*-regulated chaperon-like protein gene of EPEC. Two sites were found to be putative replication regions, one of that is similar to RepFIIA of p307 or F and the other to RepFIB of NR1. In addition to these, a third region was identified that contains plasmid maintenance genes. Insertion elements were scattered throughout the plasmid indicating the mosaic nature of the EAF plasmid and suggesting evolutionary events by which virulence genes may have been obtained.

TRANSCRIPTIONAL REGULATION OF (α2→8) LINKED POLYSIALIC ACID CAPSULE OF SEROGROUP B *NEISSERIA MENINGITIDIS*

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Capsular polysaccharide is an important virulence factor in many Gram-negative pathogens. Serogroup B *Neisseria meningitidis* produces a homopolymeric capsule composed of (α2→8) linked sialic acid that provides survival advantages during transmission and infection of meningococci, thereby helping meningococci evade host immune defense. Capsule production can be switched on or off during the infection process, indicating that environmental cues may be utilized for its regulation. Divergently transcribed biosynthesis (*synABCD*) and transport (*ctrABCD*) operons mediate the production of capsule. The intergenic overlapping promoter region of these two operons is potential site for transcriptional regulation. Transcriptional fusions of β-galactosidase reporter gene were constructed and specific deletions made within the intergenic region. The data indicated that the transport operon is constitutively transcribed at low level and no feedback regulation was observed. A potential transcriptional regulator binding site was determined and the putative regulatory factor(s) was shown to be absent in another (α2→8) linked polysialic acid capsule producing pathogen, the *E. coli* K1 strain.

ROLE OF SECRETED ANTIBODIES IN MURINE RESISTANCE TO *SALMONELLA ENTERICA* VAR TYPHIMURIUM

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Typhoid fever is an important bacterial disease of humans causing approximately 600,000 deaths each year, from 16 million infections world-wide. The disease results from an oral infection by *S. enterica* var Typhi which invades from the gastrointestinal tract through the mucosal epithelium and can be prevented by vaccination with whole killed *S.* Typhi, the purified *S.* Typhi Vi capsular polysaccharide, both injected parenterally, and by oral immunisation with the live attenuated *S.* Typhi Ty21a. One of the number of arguments used in support of the oral Ty21a vaccine is that oral immunisation will elicit mucosal immunity which may assist in providing more complete protection against *S.* Typhi, and perhaps reduce dissemination of the pathogen. We have investigated the impact of mucosal immunisation on vaccine-mediated protection in a novel knockout mice lacking the polyimmunoglobulin receptor ($pIgR^{-/-}$). The $pIgR^{-/-}$ mouse was constructed on a C57BL/6 background using BL/6 ES cells and animals lacking the pIgR had fecal antibody levels too low to be detected. The vaccine used in these studies was BRD509, an aroA/aroD mutant of the mouse virulent *S.* Typhimurium SL1344. BRD509, when fed orally to mice, protects against lethal challenge by SL1344. BRD509 was fed to C57BL/6 mice and serum and fecal samples collected for serological analysis. Serum and fecal IgA antibodies specific for *S.* Typhimurium LPS increased at the time animals become resistant to reinfection. These antibodies inhibited entry of *S.* Typhimurium into epithelial cells in *in vitro* invasion assays suggesting that a functional mucosal response may be elicited by oral immunisation with BRD509. Challenge studies in BRD509-immunised $pIgR^{-/-}$ mice revealed that the animals were immune to lethal challenge infection by SL1344, and that entry into the Peyer's patches early after infection (eg. 1-3 days) was only slightly elevated in the animals which could not secrete IgA (ie. $pIgR^{-/-}$ mice), compared with normal animals. This suggests that secretory IgA plays only a minor role, if any, in the protection of mice against mucosal infection by *S.* Typhimurium. The ItyR genotype from DBA/2 mice is being bred onto the $pIgR^{-/-}$ C57BL/6 background to determine whether mucosal antibodies play a more important role in protecting animals that perhaps more closely resemble humans in their response to *S. enterica* enteric fever. These animals should provide valuable insight into the potential importance of mucosal immunity in typhoid fever and support, or obviate, the need for oral vaccine approaches.

BURKHOLDERIA CEPACIA, AN OPPORTUNISTIC PATHOGEN THAT RESIST KILLING BY CATIONIC PEPTIDES, ESCAPES INTRACELLULAR KILLING BY MACROPHAGES AND AMOEBAE

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Burkholderia cepacia is an opportunistic pulmonary pathogen that has emerged as a serious health risk in patients with cystic fibrosis (CF). We have recently shown that several strains of *B. cepacia* can escape intracellular killing by free living amoebae (Marolda *et al.*, Microbiology, in press). In this work, we demonstrate that *B. cepacia* can survive in phagocytic vacuoles of PU5-1.8 murine macrophages for a period of at least five days without significant bacterial replication, and in the presence of macrophage activation. Bacterial entry into macrophages stimulated production of TNF-α, and primed them to release toxic oxygen radicals. Furthermore, infected macrophages primed with IFN-γ produced less nitric oxide than IFN-γ-primed uninfected cells. We propose that the ability of *B. cepacia* to resist intracellular killing by phagocytic cells may play a role in the pathogenicity of cystic fibrosis lung infection. Our data are consistent with a model where repeated cycles of phagocytosis and cellular activation without bacterial killing, may promote a deleterious inflammatory response causing tissue destruction and decay of lung function. We hypothesized that the mechanism of intracellular survival depends at least in part from the pathogen's natural ability to resist killing by cationic peptides. We have constructed and characterized transposon mutants that became sensitive to the cationic peptide polymyxin B. These mutants were unable to survive within amoebae and macrophages suggesting that several genes are associated with the property of intracellular survival. We propose that intracellular survival of *B. cepacia* within professional phagocytes may be an important factor for long-term survival in the environment, and may also play a role in the pathogenesis of lung infections in CF patients.

IDENTIFICATION OF MHC CLASS I ACCESSIBLE PROTEINS OF SALMONELLA TYPHIMURIUM THAT ARE REQUIRED FOR SURVIVAL INSIDE MACROPHAGES.

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Macrophage survival is a critical virulence mechanism of *Salmonella typhimurium* as mutants that do not survive within macrophages are attenuated for mouse virulence. To survive inside macrophages, *Salmonella* may secrete proteins into the cytoplasm. Due to their cytoplasmic localization, we have termed these proteins Class I Accessible Proteins (CAPs) since they may be subject to proteolytic degradation via the proteasome. Subsequent presentation of these peptides in context of Major Histocompatibility Complex (MHC) Class I may lead to recognition by a cytolytic T-cell (CTL). We have utilized this particular aspect of the intracellular life cycle of *S. typhimurium* to develop a powerful genetic selection, Disseminated Insertion of Class I Epitopes (DICE, Ellefson *et al.*, 1999), to identify CAPs of *S. typhimurium* that are required for survival inside macrophages.

ISOLATION AND CHARACTERIZATION OF *HAEMOPHILUS INFLUENZAE* GENES INDUCED UPON INTERACTION WITH LUNG EPITHELIAL CELLS.

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Nontypeable *Haemophilus influenzae* (NTHi) infects the lower respiratory airways of chronic obstructive pulmonary disease (COPD) patients. NTHi adhere to and enter the bronchial epithelial cell layers in a dynamic process: cells react through cytokine production, whereas the bacterium synthesizes new proteins to adapt to the environment.

We set up a system to isolate genes that are induced upon interaction with epithelial cells. These were identified by their induced promoter activity, using *cat* as a reporter. A genomic library of 8000 clones of a NTHi strain, isolated from a COPD patient, was constructed in *H. influenzae* Rd and used to infect the human lung epithelial cell line NCI-H292. Bacteria containing an induced promoter survived chloramphenicol treatment and were isolated and discriminated from constitutive promoters by replating them on plates with or without chloramphenicol.

Selection resulted in isolation of 45 induced clones, of which part of the genomic inserts were sequenced. Database search revealed that 42 clones showed homology to the sequenced Rd genome. These clones include promoters of genes encoding outer membrane proteins, stress proteins, transcription regulators and proteins of metabolic routes apparently expressed during interaction with epithelial cells. Clones also contained open reading frames, which in the Rd genome appeared to contain frame-shifts. Finally, three clones contain sequences not homologous to Rd, indicating novel genes, probably involved in NTHi virulence. Further research will focus on conditions relevant for the infection route of *Haemophilus influenzae*.

EXPRESSION OF THE PATHOGENIC AFA-3 OPERON

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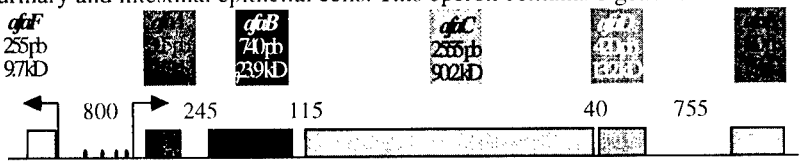
The afa-3 operon give to the *E. coli* bacterium the power to adhere and invade urinary and intestinal epithelial cells. This operon contains 6 genes :



In vitro transcription experiment shows that this region is transcribed from two divergent promoters. We constructed transcriptionnal fusions cloned on a monolysogen lambda to study the influence of several proteins on the expression of the rigth side of the operon (see figure1). A phase variation is induced by the presence of the *afaF* gene on the fusion. This means that, in a same liquid culture coexists two cell populations. The first one expresses the rigth side genes (called phase ON), whereas the second does not (called phase OFF). AfaF seems also to be a inhibitor of the transcription of these genes. S1 experiment confirms this result. Indeed, the RNA bearing *afaA* is almost undetectable in presence of AfaF, whereas it is at least 50 time more abundant in absence of this protein.

LRP protein is also necessary for phase variation. This protein acts either as an activator of the operon in the absence of AfaF or as an inhibitor when AfaF is synthesized. Repression by an unidentified effector may explain why the rigth side promoter which is silent in the absence of both AfaF and LRP *in vivo* is active *in vitro*.

INTERNALIZATION AS WELL AS FORMATION OF STRESS FIBERS DURING *BARTONELLA BACILLIFORMIS* INFECTION OF HUVECs REQUIRES RHO ACTIVATION

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Verruga peruana, the tissue phase of *Bartonella bacilliformis* infection, is characterized by pronounced cutaneous endothelial cell proliferation, resulting in characteristic skin eruptions of papules and nodules known as verrugas. Little is known concerning the biological effects of infection by *B. bacilliformis* on endothelial cells. Human umbilical vein endothelial cells (HUVEC) were infected with the *B. bacilliformis* carrying a GFP (Green Fluorescent Protein) plasmid. Using confocal microscopy we have monitored invasion of the bacteria and have demonstrated their intracellular presence up to a week following infection. Progression of infection, by fluorescent bacteria was monitored by flow cytometric analysis. An observed increase in the fluorescent intensity of the infected endothelial cells with time indicated continuous internalization and/or multiplication of bacteria inside endothelial cells. After 24 hrs of infection, phalloidin-fluorescein staining revealed changes in the actin cytoskeleton including thickening of stress fibers which unidirectionally orient along the long axis of cells. Our experiments suggests that internalization, as well as establishment of infection, leading to stress fiber formation, is Rho (a member of Ras superfamily of small GTP-binding proteins) - dependent. Pretreatment of endothelial cells with C3 exoenzyme (a bacterial toxin from *Clostridium botulinum* which inactivates Rho by ADP-ribosylation) abolished the entry of *B. bacilliformis* into these cells. Treatment of pre-infected endothelial cells with C3 exoenzyme showed inhibition of actin stress fibers formation which diminished as the time interval between infection and C3 addition increased. Loss of cell-cell contacts, perhaps due to the retraction of stress fibers, was observed using anti-PECAM antibody immunofluorescent staining or phalloidin-fluorescein staining. This infection-dependent loss of cell-cell contacts was inhibited by C3 exoenzyme mediated inactivation of Rho. Also, infected endothelial cells were demonstrated to have a considerably reduced rate of cell migration either in an *in vitro* wound or spontaneous migration assay. Furthermore, infected cells showed increased cell-matrix focal contacts, as demonstrated with anti-paxillin (a focal adhesion protein), anti-focal adhesion kinase or anti-phosphotyrosine immunofluorescent staining. These observations indicate that *B. bacilliformis* infection of endothelial cells results in marked changes in cell-cell and cell-matrix contacts, which may play an important pathogenetic role in the formation of verrugas..

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THE ROLE OF H-NS IN THE ENVIRONMENTAL REGULATION OF THREE FIMBRIAL OPERONS IN *ESCHERICHIA COLI*

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Environmental stimuli are known to regulate virulence gene expression in a variety of bacterial pathogens. In this work, a comparative study was completed to determine the influence of various environmental stimuli on the transcription of three different fimbrial operons in *Escherichia coli* and to determine the role of H-NS in this environmental regulation. The fimbrial operons studied included the *pap* operon, which encodes pyelonephritis-associated pili (P pili), the *daa* operon, which encodes F1845 fimbriae, and the *fan* operon, which encodes K99 fimbriae. P pili are associated with *E. coli* that cause upper urinary tract infections whereas *E. coli* expressing F1845 and K99 fimbriae are associated with diarrheal disease in humans and in calves and lambs, respectively.

Using *lacZYA* transcriptional fusions within each of the fimbrial operons, the effect of temperature, osmolarity, rich medium, carbon source, and amino acids were tested for their effect on fimbrial gene expression. For all three operons, low temperature (23°C), glucose as a carbon source, and rich medium (Luria-Bertani) were shown to repress transcription. High osmolarity (300 mM NaCl) significantly repressed *fan* and *pap* transcription, whereas *daa* transcription was only slightly repressed by high osmolarity. In contrast, the addition of casamino acids to M9 minimal medium did not significantly affect transcription of these operons.

H-NS (histone-like nucleoid structuring protein) is a DNA binding protein that has been shown to control transcription of a number of environmentally regulated genes in *E. coli* and other Gram- bacteria. To determine if the repressive effects of the stimuli above were mediated by H-NS, transcription was measured in an *hns* mutant strain in response to each of the different environmental stimuli. For the strain containing the *fan* transcriptional fusion, the introduction of the *hns* mutation relieved the repressive effect of low temperature, glucose, high osmolarity, and rich medium. For the *daa* operon, the introduction of the *hns* mutation relieved the repression due to low temperature, osmolarity, and rich medium, but only partially relieved the repression of transcription due to glucose. Significantly different results were observed with the *pap* operon. In the absence of a change in environmental conditions, the introduction of *hns* mutation alone reduced *pap* transcription approximately 5-fold. This result confirms earlier experiments indicating that the H-NS protein plays a positive role in *pap* transcription at 37°C. In this same strain, transcription at low temperature was higher than that seen in the wild type strain at 23°C, confirming the role of H-NS in thermoregulation of the *pap* operon. The mutation in *hns* only partially relieved repression by glucose and high osmolarity on *pap* transcription.

Together, these data indicate that low temperature, high osmolarity, glucose as a carbon source, and rich medium are important environmental cues that regulate fimbrial gene expression in *E. coli* and that H-NS is an important environmental regulator for all three of these fimbrial operons in response to several environmental stimuli.

CHARACTERIZATION OF BACTERIAL VIRULENCE MECHANISMS USING A PSEUDOMONAS AERUGINOSA-CAENORHABDITIS ELEGANS PATHOGENESIS MODEL

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Pseudomonas aeruginosa is an opportunistic bacterial pathogen with the capacity to cause a wide range of infections in both plants and animals using a shared subset of virulence factors. To identify and study bacterial virulence factors and corresponding host defense mechanisms our laboratory recently developed a pathogenesis system based on antagonistic interactions between a human clinical isolate of *P. aeruginosa*, UCBPP-PA14 (PA14), and the nematode *C. elegans*. Two mechanistically distinct modes of nematode killing are observed when PA14 is provided as a food source, one of which is mediated by the secretion of diffusible toxins and results in killing within 24 hours ("fast killing"). To identify bacterial factors required for the virulence of PA14, a genetic screen was conducted by testing 3300 PA14::Tn*phoA* insertion mutants in the fast killing model. Seven mutants were identified that displayed an attenuated fast killing phenotype, four of which also exhibited a reduced pigment phenotype as a result of decreased pyocyanin production. Pyocyanin belongs to a group of tricyclic secondary metabolites known as phenazines that are thought to exert their toxic effect through the generation of oxidative stress. Indeed, a correlation was found between the ability of *C. elegans* mutants to tolerate oxidative stress and the resistance to fast killing, demonstrating that phenazines are essential mediators of this killing process. Furthermore, C. elegans mutants lacking P-glycoproteins (ATP-binding cassette transporters) are effectivily killed by PA14 mutants that produce wild-type levels of pyocyanin.
Further characterization of the PA14::TnphoA insertion mutants will be presented here.

LISTERIA MONOCYTOGENES PHOSPHOLIPASE C (PLC) ACTIVITIES INDUCE PKC, CALCIUM SIGNALING, AND ACTIVATE HOST PHOSPHOLIPASES IN MACROPHAGES

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Listeria monocytogenes, a food borne bacterial pathogen, secretes a phosphatidylinositol PLC (PI-PLC) and a broad range PLC (BR-PLC), and listeriolysin O (LLO), a pore-forming cytolysin. PI-PLC has been shown to play a role in escape from the primary phagocytic vacuole of a macrophage, and both PLCs function in escape from the double membrane vacuole formed after cell-to-cell spread. Single mutants in the PLCs show small decreases in mouse virulence, but a mutant in both PLCs has a mouse LD_{50} approx. 500 times greater than wild type (WT) (Smith, G.A. *et al.* Infect. Immun., **63**, 4231-37, 1995). LLO mutants do not escape from the macrophage phagocytic vacuole, and are avirulent in mice. In order to study potential effects of bacterial PLCs on host intracellular signaling, we have examined the effects of infection of J774 murine macrophage-like cells with WT and mutants on intracellular Ca^{2+} and have observed an increase in cytosolic Ca^{2+} $[Ca^{2+}]_i$ within 1 min after infection which is dependent on PI-PLC and LLO, but not on the BR-PLC. This Ca^{2+} elevation is inhibited by the receptor-operated Ca^{2+}-channel blocker SK&F96365. After declining to near background levels, there is a brief 2nd, and a more prolonged 3rd increase in $[Ca^{2+}]_i$ to ~ 0.8 μM. These arise from release of calcium from intracellular stores and the last elevation occurs during the internalization phase of infection. Only an attenuated late calcium-signal was seen with a PI-PLC mutant. An LLO mutant produced no calcium signaling. WT and BR-PLC mutants were delayed in associating with and entry into J774 cells, whereas LLO and PI-PLC mutants were taken up very rapidly. Pretreatment of J774 cells with SK&F96365 resulted in more rapid uptake of the WT and diminished ability to escape from the primary vacuole (Wadsworth, S.J. and Goldfine, H, Infect. Immun. **67:** 1770-78, 1999). Our recent studies have shown that PKCδ is rapidly activated in WT-infected cells, but not in cells infected with a PI-PLC mutant. PKCδ appears to play a role in opening the calcium channel(s). We have studied bacterial and host phosphoinositide-specific PLC activities early after infection in J774 cells. In cells prelabeled with ^{3}H-inositol (Ins) we observe a time-dependent increase in ^{3}H-Ins-P beginning within 10 min after infection, which is severely reduced when cells are infected with either a mutant in PI-PLC or in LLO. In J774 cells, Ins-P_3, a product of **host** PLCs, also increases after infection, and this increase is partially dependent on the presence of bacterial PI-PLC and completely dependent on LLO. Data will be presented that indicate a role for the sphingomyelinase activity of *L. monocytogenes* BR-PLC in generating calcium signaling. We have also observed activation of host phospholipase D after infection with *L. monocytogenes*. Among the secreted proteins, only LLO appeared to be required for PLD activation. Thus phospholipases working with LLO are important in modulating early uptake of *L. monocytogenes* and its eventual fate in a macrophage (Supported by an NIH grant).

VIBRIO CHOLERAE BIOFILM DEVELOPMENT

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We are interested in understanding the impact that the ability to form a biofilm has on the emergence of human pathogens that are also natural inhabitants of the aquatic environment. *Vibrio cholerae*, a gram-negative rod responsible for the severe diarrheal disease known as cholera, is a prototype of such pathogens. A study of the genetic basis of biofilm development by *V. cholerae* has been undertaken to begin to unravel the role played by biofilm formation in the emergence of this pathogen. Our results demonstrate that *V. cholerae* El Tor does not use the virulence-associated pilus TCP to form biofilms on an abiotic surface. Instead, the MSHA pilus and flagellar motility are required for attachment to the surface. Furthermore, exopolysaccharide is necessary for generating and stabilizing the three-dimensional biofilm structure. These data suggest that the cell surface structures required for adhesion to the intestinal epithelium and abiotic surfaces are distinct. A genetic and structural comparison of the biofilms made by the three epidemic types of *V. cholerae* is underway.

DIFFERENTIATION OF MONOCYTES TO MACROPHAGES SWITCHES THE *MYCOBACTERIUM TUBERCULOSIS* EFFECT ON HIV-1 REPLICATION FROM ACTIVATION TO REPRESSION: MODULATION OF IFN RESPONSE AND C/EBP

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HIV-1 replication is suppressed in uninflamed lung macrophages and increased during tuberculosis. Attempts to model the activation of HIV-1 replication in monocytes and macrophages *in vitro* have produced opposite results depending on the state of differentiation of the cells. After infection with *M. tuberculosis*, monocytes enhance HIV-1 replication and produce a stimulatory 37 kDa C/EBPβ transcription factor while macrophages suppress HIV-1 replication and produce an inhibitory 16 kDa C/EBPβ transcription factor. Interferon (IFN)-β induces inhibitory 16 kDa C/EBPβ in macrophages but has no effect on C/EBPβ expression in monocytes. Macrophages but not monocytes are able to activate ISGF-3, a type I IFN (IFNα/β) specific transcription factor, after infection with *M. tuberculosis* or stimulation with type I IFN. Both monocytes and macrophages maintain interferon responsiveness, activating STAT-1 homodimer formation and transcription of the Stat-1 gene after IFN stimulation. Both monocytes and macrophages are able to activate the NF-κB p65 cRel transcription factor after an inflammatory stimulus, indicating C/EBP mediated transcriptional repression induced by type I IFN in macrophages is dominant over the stimulatory effect of NF-κB. Therefore, differentiation of monocytes to macrophages modulates the interferon response. Only macrophages are able to respond to infection with *M. tuberculosis* or stimulation with type I IFN by producing ISGF-3, inducing the 16 kDa C/EBPβ transcriptional repressor and suppressing HIV-1 replication via a transcriptional mechanism.

ENDOTHELIAL CELL RESPONSES ON *CHLAMYDIA PNEUMONIAE* INFECTION

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Atherosclerosis is an inflammatory disease. An association of *C. pneumoniae* infection with atherosclerosis and coronary heart disease (CHD) has been suggested by epidemiological studies and by the detection of *C. pneumoniae* organisms in atherosclerotic lesions in both humans and animal models of atherosclerosis. Previously, it has been shown that *C. pneumoniae* is capable of replicating in cell types found with atheromatous lesions: endothelial cells, smooth muscle cells and monocytes/macrophages, yet the role of *C. pneumoniae* in the pathogenesis of atherosclerosis has not been determined. Since injury-caused dysfunction of endothelial cells leads to compensatory responses that alter the normal homeostatic properties of the endothelium, we investigated whether *C. pneumoniae* infection of human umbilical vein endothelial cells (HUVEC) alters the gene expression pattern of this particular cell type. Therefore, we apply differential display reverse transcription PCR to identify differentially expressed transcripts in *C. pneumoniae* infected HUVE cells. With different sets of primers we identified, isolated and sequenced several fragments of known and unknown genes, either expressed or repressed in infected cells versus noninfected cells. Results will be presented. Since it is known that injury induces endothelial cells to have procoagulant instead of anticoagulant properties, we investigated thrombomodulin (TM) and tissue factor (TF) as well as other hemostasis factors gene expression in infected endothelial cells. Our results demonstrate that infection with *C. pneumoniae* leads to repression of the anticoagulant protein thrombomodulin on endothelial surface. The unbalanced hemostatic properties of the endothelium in response to *C. pneumoniae* infection towards procoagulatory events may lead to an increase in local thrombogenicity and coronary heart disease.

T LYMPHOCYTE RESPONSE TO *NEISSERIA GONORRHOEAE* PORIN (POR) IN INDIVIDUALS WITH MUCOSAL GONOCOCCAL INFECTIONS.

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This study characterizes the anti-Por T cell response in patients with urogenital gonococcal disease to determine whether mucosal gonococcal infection can generate circulating T lymphocytes which are Por specific. Patients with urogenital gonococcal disease can generate a humoral response towards gonococcal outer membrane components, including Por. As Por is a protein, the anti-Por antibody response is T cell dependent. T lymphocytes from a majority of the patients examined, obtained at initial diagnosis of urogenital gonococcal infection, proliferated upon incubation with Por, as compared to minimal induced proliferation of T lymphocytes from normal volunteers. Using intracellular cytokine staining and flow cytometric analysis, we determined that a significant increase in IL-4 producing CD4+ T helper lymphocytes was seen in these patients upon incubation with Por, while no increase in IL-4 producing CD4+ T lymphocytes was observed in normal volunteers. Interestingly, the same trend was observed in CD8+ T lymphocytes from these patients. There was no measured increase in IL-2, IL-10, IL-12, IFN-_, and TNF-_ production by T lymphocytes. Concomitant increases in IL-4 production in T lymphocytes that could potentially traffic to mucosal surfaces (expressing the mucosal addresin, VLA _4/ _7, on their surface) upon Por incubation were also observed, but the increases were similar in T lymphocytes that were VLA_4/_7 negative. In conclusion, mucosal gonococcal disease can induce Por specific circulating T lymphocytes, with a Th2 phenotype, and a portion of these Por specific T lymphocytes can potentially traffic to mucosal surfaces.

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SALMONELLA GALLINARUM AND *SALMONELLA PULLORUM* ENCODING *SALMONELLA TYPHIMURIUM* TYPE 1 FIMBRIAE EXHIBIT INCREASED INVASIVENESS FOR MAMMALIAN CELLS

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S. gallinarum and *S. pullorum* are *S.enterica* biotypes that exhibit host specificity for poultry and aquatic birds. Normally these serotypes are not capable of causing disease in mammalian hosts. Interestingly, during their evolution toward host restriction *S. gallinarum* and *S. pullorum* lost both flagellar expression and their ability to mediate mannose-sensitive hemagglutination (MSHA), and it has been speculated that the loss of these two functions resulted in the loss of virulence of *S. gallinarum* and *S. pullorum* in mice and humans. We examined whether the restoration of the ability to exhibit MSHA to these strains would also increase their ability to invade mammalian cells in a tissue culture model and a murine ligated ileal loop model of infection.

S. gallinarum and *S. pullorum* were transformed with a plasmid encoding the *S. typhimurium* LT2 type 1 fimbrial operon. The transformants exhibited hemagglutination of guinea pig erythrocytes and also showed a 20- to 30-fold increase in the ability to adhere to the human epithelial HEp-2 cell line. Invasion assays indicated that this enhanced ability to adhere to HEp-2 cells also correlated with a 20- to 60-fold increased invasion efficiency. The presence of 0.3% mannose decreased adherence and invasion levels similar to *S. gallinarum* and *S. pullorum* strains transformed with the vector alone. In a murine ligated ileal loop model, a 32% increase in the number of M cell ruffles was seen when *S. gallinarum* encoded the *S. typhimurium* type 1 fimbrial operon. Current experiments are directed at determining whether the increased ability of the transformed *S. gallinarum* and *S. pullorum* strains to invade mammalian cells is dependent upon a functional type III secretion system encoded by the SPI 1 pathogenicity island present in both *S. gallinarum* and *S. pullorum.*

HOST CELL SURFACE HEPARAN SULFATE IS CRITICAL FOR THE INFECTIVITY OF *CHLAMYDIA PNEUMONIAE*

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Chlamydia pneumoniae is an obligate intracellular parasite which has been linked to atherosclerosis. The mechanism of host cell infection by *C. pneumoniae* is not understood. This study examined the role of glycosaminoglycans (GAGs) for the infectivity of *C. pneumoniae*. Soluble heparin or heparan sulfate inhibited the infectivity of *C. pneumoniae* competitively, whereas other GAGs had no effect. Pre-treatment of *C. pneumoniae* or host cells with heparin prior to the infection demonstrated that the reduction of the infectivity resulted from binding of heparin to the organism. In contrast, heparin pre-treatment of *C. trachomatis L2* did not reduce the infectivity of the organism. Enzymatic removal of heparan sulfate from the surface of host cells led to a 10-fold decrease of *C. pneumoniae* infectivity. Mutant chinese hamster ovary cell lines defective in heparan sulfate synthesis were 10-fold less susceptible to *C. pneumoniae* infection than the wild-type cell line. In conclusion, results of this study strongly suggest that host cell surface heparan sulfate serves as a receptor for *C. pneumoniae*. This mode of GAG-dependent infectivity of *C. pneumoniae* is different from the mechanism of attachment described for other chlamydia species and may contribute to the differences in tissue tropism.

ISOLATION OF RESTRICTION ENDONUCLEASES FROM HELICOBACTER PYLORI

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H. pylori are bacteria that colonize the human stomach. This colonization increases risk for peptic ulcer disease and gastric adenocarcinoma. Restriction-modification systems in H. pylori are not well studied. DNA analysis of the whole genomic sequences of H. pylori strain J99 and 26695 predicts that the strains have 14 or 15 type II R-M systems, an unusually high number. To investigate R-M systems in H. pylori, we sought to isolate restriction endonucleases from various H. pylori strains. By column chromatography, we were typically able to identify two to six endonuclease activities per strain. In H. pylori strain 60190 we identified four endonucleases: HpyII (MboII isoschizomer, recognition site GAAGA), HpyIV (HinfI, GANTC), HpyV (TaqI, TCGA), and HpyVIII (HpaII, CCGG). In strain J178 we found four endonucleases, including one with a novel specificity: Hpy178II (MboII, GAAGA), Hpy178III (new phenotype, TCNNGA), Hpy178VI (FokI, GGATG), and Hpy178VII (HaeIII, GGCC). In strain J188, we identified Hpy188III (the new phenotype found in strain J178, TCNNGA), and a second new specificity, Hpy188I (new phenotype, TCNGA). In strain CH4, we were able to identify endonucleases: HpyCH4I (NlaIII, CATG), HpyCH4II (DdeI, CTNAG), HpyCH4III (Tsp4CI, ACNGT), HpyCH4IV (MaeII, ACGT), HpyCH4V (CviRI, TGCA), and HpyCH4VI (the new phenotype found in strains J178 and J188, TCNNGA). In strain J99, three endonucleases were isolated: Hpy99I (new phenotype, CGWCG), Hpy99II (Hha I, GCGC), and Hpy99III (Tsp45I, GTSAC). Digestion of chromosomal DNA from ten H. pylori strains including strain J99 and 26695 using purified endonucleases reported above further indicates that the DNA modification status of various H. pylori strains is highly diverse. We conclude that each H. pylori strain generally has a varying complement of functioning R-M systems.

ROLE OF THE *LISTERIA MONOCYTOGENES* DNAK CHAPERONE IN MACROPHAGE-PHAGOCYTOSIS

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Listeria monocytogenes is a facultative intracellular pathogen that can escape bactericidal mechanisms and grow in macrophages. Its ability to survive in the macrophages is crucial to the pathogenesis of Listeria infections. The intracellular environment of macrophages is one of the most stressful environments encountered by an invading bacterium during course of infection. To study the role of the major stress protein, DnaK chaperone, in intracellular survival of *L.monocytogenes*, we cloned the *dnaK* gene and then constructed an insertional mutation in the gene. Molecular analysis revealed a tetracistronic structure of the operon consisting the genes *hrcA, grpE, dnaK* and *dnaJ*. The ability of the *dnaK* mutant to survive in the macrophages was examined. Immediately after phagocytosis, the number of viable mutant cells found in macrophages was significantly lower compared to that of intracellular wild type cells. However, following a 1 to 3 h latency period, the mutant multiplied in a similar fashion to the wild type in macrophage cells. A quantitative analysis of intracellular bacteria in the macrophages by microscope and a binding assay of bacteria to the surface of macrophages by ELISA revealed that the lower number of viable mutant cells in macrophages at an early stage is due to the low efficiency of phagocytosis resulting from its reduced binding capacity to the surface of macrophages. These results demonstrated that the DnaK chaperone is essential for efficient phagocytosis though it does not largely contribute to the intracellular growth of *L.monocytogenes*. Since DnaKs are believed to reside in the bacterial cytoplasms, it is unlikely that bacterial DnaK is directly involved in bacterial adhesion to the surface of macrophages. To define the bacterial component involved in the phagocytosis, we compared the cell wall proteins extracted from both wild and mutant strains. In the mutant strain, the 24 kDa protein disappeared. The molecular analysis of this protein is in progress. This is the first evidence that the DnaK chaperone is involved in the phagocytosis of bacteria with macrophages.

TRANSCRIPTION ACTIVATION BY THE *VIBRIO CHOLERAE* VIRULENCE ACTIVATOR TOXT

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Coordinate expression of many virulence genes in the human pathogen *Vibrio cholerae* is under the direct control of the ToxT protein. These include genes whose products are required for the biogenesis of the toxin-coregulated pilus (TCP) and cholera toxin (CTX). ToxT is a member of the AraC family of transcriptional activators and its mode of action is being investigated. This work examines interactions between ToxT and the promoters of *ctx* and *tcpA* genes, with the aim of defining promoter requirements and recognition sites for ToxT activation, and ultimately, the mechanism by which ToxT activates transcription.

Analysis of *ctx-lacZ* transcriptional fusions showed that a minimum of 3 direct repeats of the sequence TTTTGAT are required for ToxT-dependent activation. A 6xHis-tagged fusion of ToxT was constructed and purified for *in vitro* experiments to determine DNA binding requirements of ToxT. Electrophoretic shift experiments demonstrated that 6xHis-ToxT directly and specifically interacts with the *ctx* promoter. Preliminary DNaseI footprinting analysis of the *ctx* promoter revealed ToxT binding regions from -111 to -43 which includes 8 direct repeats. As the concentration of ToxT was increased, sites closer to the basal promoter elements also became occupied. Analysis of *tcpA-lacZ* transcriptional fusions showed that the region from -85 to -28 of the *tcpA* promoter contains elements that are responsive to ToxT-dependent activation. There is no apparent sequence similarity between the *ctx* and *tcpA* promoters, but they are both A-T rich, so it is likely that ToxT binds to and activates transcription from an A-T rich region.

IDENTIFICATION OF *S. pneumoniae* GENES ESSENTIAL FOR *in vitro* GROWTH OR FOR PATHOGENICITY

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The appearance of methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant streptococci and vancomycin-resistant enterococci as well as multiple drug resistance in other clinically important bacteria has created an urgent need for new antibiotics. Genome sequences are a catalog of all of the possible targets that can be exploited for therapeutic intervention. If new classes of chemical entities having antibiotic activity are to be found, an antimicrobial target for high throughput inhibition screening must be novel and possess the appropriate spectrum and selectivity. The gene products should also be essential for cell viability *in vivo*. As this is difficult to establish we have ascertained their transcription in infection models and subsequently determined their essentiality *in vitro* and *in vivo* to indicate their utility as targets.

Whole genome comparative sequence analysis has been used to prioritise all *S. pneumoniae* open reading frames according to their presence in a range of bacteria and their absence in eukaryotes. Knowledge of every gene sequence has allowed the transcription analysis of over 500 broad spectrum *S. pneumoniae* genes in a respiratory tract infection model in the mouse. Subsequently, essentiality studies of these transcribed genes have identified a number indispensable for cell viability *in vitro* or for pathogenicity.

Biochemical and Genetic Characterizations of a novel cytotoxic protease from Coagulase-Negative Staphylococci of bovine mastitis

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Coagulase-Negative Staphylococci (CNS) have been considered as the minor pathogens of bovine mastitis. However, many studies have shown that some CNS could cause acute intra-mammary infections and chronic production losses in dairy cows. The Animal Diagnostic Lab of the Pennsylvania State University has spent several years on an ongoing CNS research project. The early study indicated that *S. chromogenes* was the most common species isolated from bovine mastitis samples. Our research goals are to demonstrate a potential virulence factor of *S. chromogenes* and to elicit its functions via *in vitro* and *in vivo* studies. Using several established cell lines and primary goat mammary epithelial cells for *in vitro* cytotoxic study, bacterial supernatants of most *S. chromogenes* strains caused cell rounding and detachment from culture plates. A 34-36 kDa protein was responsible for cell rounding and detachment. Protein peptide sequencing and protein mass analysis indicated that it was a novel metalprotease. This novel protease is a heat-labile and has strong casein hydrolysis activity. The proteolytic activity of the novel protease could be inhibited by metal and zinc chelator such as EDTA and 1,10-penanthroline but not inhibited by serine protease inhibitor such as PMSF. The protease had an optimal pH at 7.2-7.5 for the proteolytic activity and had a pI of 5.4 on an IEF gel. Two degenerate primers were made according to the sequence information of two short peptides of the 34-36 kDa protease, and a 480 bp PCR product was sequenced. Two long PCR primers were designed based on the 480 bp sequence for circular PCR, and the full length of the 34-36 kDa protease gene was sequenced. The sequence data indicated a 1503 bp long open reading frame (ORF). The ORF encodes a 501 amino acid pre-pro-protease, which has 25 amino acid signal sequence and 176 amino acid pro sequence. According to the N-terminal sequence of the mature protease, the protease has 300 amino acid with a predicted pI about 5.21 and predicted molecular weight about 33.3 kDa. The mature protease has a zinc binding motif AHEITH and share structural homology with many metalproteases. A 2.0 kb PCR fragment encoding the pre-pro-protease has been cloned into the pGEM3Zf vector in *E. coli* JM109 and pHY300PLK vector in *Staphylococcus aureus* strain RN4220. Our future research will focus on creation of a transposon Tn917 knock-out mutant to demonstrate the *in vivo* functions of the 34-36 kDa protease in a goat challenge study.

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MOLECULAR ANALYSIS OF GENETIC DIFFERENCES BETWEEN VIRULENT AND AVIRULENT STRAINS OF *AEROMONAS HYDROPHILA* ISOLATED FROM DISEASED FISH

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Short running title: screening virulent genes of *A. hydrophila*
Keywords: *A. hydrophila*, genomic substraction, virulence genes.

Aeromonas hydrophila, a normal inhabitant of aquatic environments, is an opportunistic pathogen of a variety of aquatic and terrestrial animals, and mammals including humans. *A. hydrophila* PPD134/91 is defined as virulent whereas PPD35/85 is defined as avirulent due to their different in LD_{50} values in fish. We employed suppression subtractive hybridization (SSH) to identify genetic differences between these two strains. Sixty-eight genomic regions of differences were absent in PPD35/85, and these DNA sequences were determined. Sixteen open reading frames (ORFs) encoded by 23 fragments showed high homology to known proteins of other bacteria. ORFs encoded by the remaining 45 fragments were identified as new proteins of *A. hydrophila* showing no significant homology to any known proteins. Among these PPD134/91-specific genes, 22 DNA fragments were present in most of the eight virulent strains but mostly absent in our seven avirulent strains suggesting that they are universal virulence genes in *A. hydrophila*. These included five known virulence factors of *A. hydrophila*: haemolysin (*hly*A), protease (oligopeptidase A), outer membrane protein (Omp), multidrug resistance protein, and histone-like protein (HU-2). Another 46 DNA fragments were mainly present in PPD134/91 indicating the biochemical heterogeneity among motile aeromonads. Some of these fragments encoded virulence determinants. These included genes for the synthesis of O-antigen and Type II Restriction Modification system. Our results indicated that SSH is successful in identifying genetic differences and virulence genes among different strains of *A. hydrophila*. These findings will provide new clues to understanding the pathogenicity of *A. hydrophila*. Universal virulence genes identified in this study will be used for the development of diagnostic kits and new therapies to combat diseases caused by motile aeromonads.

TNF RECEPTOR P55 CONTROLS THE SEVERITY OF ARTHRITIS IN EXPERIMENTAL YERSINIA ENTEROCOLITICA INFECTION

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Objective To dissect the host defense mechanisms in relation to the early stages of *Yersinia*-associated arthritis by evaluating the impact of TNFRp55 deficiency on *Yersinia enterocolitica* infection.

Methods TNFRp55-/- and C57BL/6 mice were inoculated i.v. with an arthritogenic strain 8081 of *Y. enterocolitica* serotype O:8. Mice were observed daily for generating survival curve and monitoring arthritis. In subsequent sets of experiments, mice were sacrificed at day 14 after infection for examination of histology in joints, bacterial clearance, macrophage microbicidal activity, nitric oxide production and oxidative burst generation, and cytokine production.

Results There was an 80% mortality rate in TNFRp55-/- mice compared with 25% in the controls at 8 weeks after inoculation with 70 colony forming units of *Y. enterocolitica* O:8. Histopathologic examination revealed that TNFRp55-/- mice developed more extensive and severe arthritis including cartilage degradation and bony destruction than the controls day 14 after infection. The joint pathology in TNFRp55-/- mice was correlated with the higher bacterial load in liver, spleen and lungs, and with the increased levels of IL-10. TNFRp55-/- mice displayed impaired intracellular killing of bacteria by macrophages. This was associated with decreased nitric oxide production and oxidative burst activity.

Conclusion This study demonstrates that TNF signalling via TNF receptor p55 controls the severity of *Yersinia*-induced arthritis and implicates that TNF-mediated macrophage microbicidal activity as a central event in this process.

Autoinducer Binding by the Quorum-Sensing Regulator TraR Causes Protein Dimerization, Increases Affinity for Target Promoters, and Stabilizes TraR Against *in vivo* Proteolysis.

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TraR is a LuxR-type transcriptional regulator from *Agrobacterium tumefaciens* whose activity requires the autoinducer-type pheromone *N*-3-oxooctanoyl-L-homoserine lactone. When oveproduced in *E. coli*, TraR accumulated in a soluble form only in the presence of the autoinducer. TraR-autoinducer complexes were purified to homogeneity and found to contain one autoinducer molecule per protein monomer. TraR-pheromone complexes bound with high affinity and selectivity to a predicted TraR binding site. TraR-autoinducer complexes eluted from a gel filtration column as a dimer, and also bound DNA as a dimer, explaining an observed lack of binding cooperativity. TraR-autoinducer complexes activated two promoters that flank this binding site, and expression of these promoters was elevated 30 fold by using a supercoiled template rather than a liner template.

Autonducer altered the properties of TraR in several ways. First, whereas TraR-autoinducer complexes are dimeric, apo-TraR eluted from a gel filtration column as a monomer. Second, autoinducer increased the affinity of apo-TraR for its DNA binding site. Third, autoinducer dramatically increased TraR abundance *in vivo* by causing a 20 fold decrease in TraR turnover rates, indicating that autoinducer stabilizes TraR against proteolysis *in vivo*.

RECOGNITION OF *SALMONELLA* ANTIGENS BY CD4+ T CELLS FROM PROTECTIVELY IMMUNIZED MICE. Brad T. Cookson, Lisa Cummings, Molly Brennan, and Jimmie Lara, Departments of Laboratory Medicine and Microbiology, University of Washington, Seattle, WA

Attenuated *Salmonella typhimurium* delivered orally to susceptible mice stimulates long lasting protection against lethal enteric fever caused by virulent *Salmonella*. Both B cell and T cell responses contribute to this immunity. Protectively immunized C3H/HeJ mice contain CD4+ T cells that recognize the *Salmonella* flagellar filament protein FliC and an unknown low molecular weight antigen. This latter antigen called p10, is protease-resistant, associates with LPS, and is expressed by most members of *Enterobacteriaceae*, including *E. coli*, *Enterobacter*, *Yersinia*, and *Shigella*. T cell recognition of this antigen is restricted by the class II MHC molecule Ek. p10 is found in bacterial culture supernatant and behaves as an ~ 10kD species on SDS-PAGE. In its native state, p10 from supernatant uniformly displays an apparent molecular weight of almost 80 million using size-exclusion chromatography, and electron microscopy identified membrane vesicles (MVs) approximately 100 nm in diameter. SDS-PAGE indicates MVs contain a subset of bacterial proteins. A portion of p10 is surface-exposed and susceptible to proteinase K digestion, while the stimulatory T cell epitope maps to an approximately 5 kD protease-resistant domain. These data are consistent with the hypothesis that surface-exposed proteins are the primary bacterial antigens recognized by memory CD4+ T cells from protectively immunized mice, and therefore immune responses to such antigens are likely to be important for immunity against *Salmonella*. Further, these data indicate a conserved protein present in MVs is recognized by T cells from orally immunized mice, suggesting that MVs can deliver bacterial proteins to host cells in vivo.

PLASMINOGEN-DEFICIENT MICE HAVE INCREASED RESISTANCE TO PLAGUE.

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Yesinia pestis, the causative agent of plague, produces an outer membrane protease designated Pla. This protease plays an important role in virulence. Pla$^-$ mutants of strain KIM 1000 have an LD_{50} one million-fold higher than that of wild-type when mice are infected by subcutaneous injection. Nonetheless, these mutants remain highly infectious (ID_{50} less than 10) and are also highly virulent if injected intravenously. Very few inflammatory cells are seen at foci of infection containing the wild-type bacteria, while large numbers, predominately neutrophils, accumulate at foci produced by Pla$^-$ mutants. One established activity of Pla in vitro is the proteolytic conversion of plasminogen to plasmin. Plasmin is the major protease of physiological fibrinolysis, and plasminogen is its inactive zymogen precursor. To determine if plasminogen activation by Pla contributes to the virulence of *Y. pestis*, disease in plasminogen-sufficient and plasminogen-deficient mice was compared. Results of these experiments include the following:

(1) Plasminogen-deficient mice have increased resistance to *Y. pestis* corresponding to an increase in LD_{50} of about 100-fold. This is not due to the abnormalities resulting from congenital plasminogen deficiency because it is not displayed by mice lacking endogenous plasminogen activators. These activator-deficient mice also develop the abnormalities associated with plasminogen deficiency because they cannot generate active plasmin. This result implies that plasminogen activation is a significant activity of Pla in vivo, but only partially explains the contribution of Pla to virulence.

(2) When either mice lacking plasminogen or bacteria lacking Pla are used, large numbers of inflammatory are found at foci of infection. This supports the hypothesis that Pla activates plasmin, and plasmin helps to suppress inflammation.

(3) Fibrin, formed by the action of thrombin on fibrinogen, is the major protein of blood clots and the major physiological substrate of plasmin. If degradation of fibrin by Pla-activated plasmin is an important activity during *Y. pestis* infection, the course of disease and the role of Pla should be altered in fibrinogen deficient mice. Foci of infection in mice without fibrinogen contained very few inflammatory cells. This result was independent of the plasminogen status of the mice, and also independent of Pla production by the bacteria. Thus, fibrinogen is required for the accumulation of inflammatory cells, at least in the initial stages pf plague. Nonetheless, Pla$^+$ bacteria were much more virulent than Pla$^-$ bacteria in fibrinogen-deficient mice. Degradation of fibrin by plasmin may be responsible for the poor inflammatory cell response seen in wild-type mice infected with Pla$^+$ *Y. pestis*, but cannot fully explain the contribution of Pla to virulence.

(4) If degradation of fibrin is the sole virulence enhancing activity of plasmin during *Y. pestis* infection, the plasminogen status of fibrinogen-deficient mice should not affect the course of disease. This prediction is not fulfilled: when infected with Pla$^+$ *Y. pestis*, mice lacking both plasminogen and fibrinogen survive significantly longer than do mice lacking only fibrinogen. Thus, plasmin(ogen) contributes to virulence independently of fibrinolysis.

MOLECULAR AND CELLULAR DETERMINANTS OF ANTIBODY EFFICACY AGAINST *CRYPTOCOCCUS NEOFORMANS*. Arturo Casadevall. Albert Einstein College of Medicine.

C. neoformans is a fungal pathogen that presents the immune system with an unusual combination of having a polysaccharide capsule and being capable of intracellular parasitism. Historically the role of natural humoral immunity against *C. neoformans* was uncertain because of contradictory results in experimental studies. However, several groups have now shown that monoclonal antibodies (mAbs) to the polysaccharide capsule can protect against infection in animal models. Administration of specific mAbs prolongs survival, reduces tissue fungal burden, and clears polysaccharide antigen. Antibody efficacy is dependent on mAb isotype, specificity and affinity. Furthermore, antibody efficacy is dependent on T cell function. Despite the efficacy of certain mAbs in modifying the course of infection to the benefit of the host antibody administration seldom clears the infection. We have also studied *C. neoformans* pathogenesis to understand how this fungus evades humoral immunity. Persistence of infection is not associated with loss of mAb-binding epitope. A detailed study of murine pulmonary infection reveals that this fungus is a facultative intracellular pathogen that uses its capsule to avoid killing by phagocytic cells. Intracellular survival may also be aided by melanin synthesis in the cell wall since melanin can protect against cellular microbicidal mechanisms. Phenotypic switching provides yet another potential strategy for evasion of the immune system. In summary, mAbs mediate protection by enhancing cellular immunity and but *C. neoformans* can persist in tissue by an active process by which this fungus subverts host defenses.

LEX2 ENCODES A β-GLUCOSYL TRANSFERASE THAT HAS PROFOUND EFFECTS ON BIOSYNTHESIS AND PHASE VARIATION OF LPS IN *HAEMOPHILUS INFLUENZAE.*

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Type b *Haemophilus influenzae* has the propensity to cause invasive disease such as meningitis. A major virulence determinant of *H. influenzae* is lipopolysaccharide (LPS). Importantly, LPS epitopes switch on and off (phase vary) at high frequency aiding survival of the pathogen in the human host. Three loci contributing to LPS phase variation, *lic1A*, *lic2A*, and *lgtC* have been identified. Each locus possesses tetranucleotide repeats within the 5' region. The loss or gain of repeats in these loci creates a translational switching mechanism which generates the observed phase variation of LPS.

It was reported in 1994 by Jarosik and Hansen that another locus, *lex2* contributes towards phase variation of an LPS epitope recognised by Mab 5G8. This locus comprises two *orf*s, *lex2A* and *lex2B*. Tetranucleotide repeats are present in the 5' region of *lex2A*. The precise role of *lex2* in phase variation of LPS in *H. influenzae* was investigated in three strains whose LPS structures are characterised.

In RM7004, a strain that normally translates *lex2* (permitted by an in-frame number of repeats), *lex2* was insertionally inactivated. In RM153, a strain that normally does not express *lex2* the repeats were removed to allow constitutive expression of this locus. Finally *lex2* was inserted by recombination into RM118 (Rd), a strain that naturally lacks this locus. The LPS of all three modified strains was characterised by SDS-PAGE, Mab reactivity, mass spectrometry and NMR. The findings demonstrate that *lex2* encodes a β-glucosyl transferase which adds a second glucose to the first β-glucose extending from the first heptose of *H. influenzae* LPS. Importantly, this second glucose completes a stub for addition of a phase variable digalactoside. This particular digalactoside was found to bind Mab 5G8 strongly. Addition of this digalactoside appears to be propagated by the same loci, *lic2A* and *lgtC* that add a digalactoside as a terminal extension from the second heptose. Constitutive expression of the digalactoside extending from the second heptose was achieved by removing the repeats from *lic2A* and *lgtC* rendering these two genes permanently in-frame. Further removal of the repeats from *lex2* allowed constitutive expression of both digalactosides. The biological significance of expressing one or two digalactosides was investigated in RM153. The killing affect of normal human serum and intravascular survival in the infant rat (after IP inoculation) were determined. A significant increase in serum resistance was found for RM153 constitutively expressing one digalactoside and an even greater increase in resistance to both rat and normal human serum was demonstrated for RM153 constitutively expressing two digalactosides.

LPS AFFECTS RESPONSIVNESS OF MACROPHAGES TO IFNg BY MODULATING THE ACTIVITY OF STAT1.

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Interferon gamma (IFNg) is a potent inducer of macrophage activation. It exerts effects on gene expression through activation of the transcription factor STAT1. STAT1 activity was shown to be regulated by tyrosine as well as serine phosphorylation. The tyrosine phosphorylation is required for dimerization, nuclear translocation and DNA binding while the serine phosphorylation is essential for maximal transactivation.

LPS was found to rapidly induce serine phosphorylation of STAT1 and thereby it enhanced the activation of STAT1 by IFNg. Here we show that the LPS-induced serine kinase is inhibited by the p38 MAP kinase inhibitor SB203580. We were not able to detect phosphorylation of STAT1 by p38 in vitro. Also, the kinetics of the serine phosphorylation of STAT1 is slower as compared to activation of p38 by LPS. Therefore we tend to speculate that another kinase downstream of p38 is responsible for the serine phosphorylation of STAT1. Interestingly, the STAT1 serine kinase induced by IFNg is not sensitive to SB203580 which implicates that the IFNg and LPS acivated signaling cascades may differ at least upstream of the STAT1 serine kinase.

Furthermore, we have found that a longer than 4 hour treatment of macrophages by LPS suppresses the activation of STAT1 by IFNg by reducing the IFNg mediated tyrosine phosphorylation of the transcription factor. Consistently, the activity of the IFNg regulated tyrosine kinase JAK1 and the transcription of a STAT1 dependent reporter gene was decreased as well. A detailed examination of the signaling events revealed that LPS directly activates the expression of the suppressor of cytokine signaling3 (SOCS3) protein which is known to have a negative effect on IFNg signaling path. These findings provide a link between the deactivating effect of chronic LPS exposure on macrophages and the ability of LPS to induce SOCS3.

INHIBITION OF T AND B LYMPHOCYTE ACTIVATION AND FUNCTION BY A *YERSINIA PSEUDOTUBERCULOSIS* VIRULENCE FACTOR – YOPH

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Numerous investigators have shown that the clearance of *Yersinia* is dependent on the specific immune response. Mice lacking T cells are exquisitely sensitive to *Yersinia* infection, and a humoral response to *Yersinia* can be protective. Given the key role of the specific immune system in the clearance of *Yersinia*, we asked if the bacteria have evolved mechanisms to directly affect T and/or B cells. After a transient one-hour exposure to *Y. pseudotuberculosis*, T and B cells were significantly impaired in their ability to become activated through their antigen receptor. T cells exposed to wild type *Y. pseudotuberculosis* were inhibited in their ability to produce cytokines, flux calcium, and signal via tyrosine phosphorylation upon T cell receptor stimulation. B cells exposed to *Yersinia* were unable to upregulate surface expression of the co-stimulatory molecule, B7.2, and to signal via tyrosine phosphorylation upon B cell receptor ligation. Through the use of *Y. pseudotuberculosis* mutants, we demonstrated that the inhibitory effect in both T cells and B is dependent on the production of YopH, a tyrosine phosphatase. Additionally, we show that after intravenous infection of mice with wild-type *Y. pseudotuberculosis*, but not with a YopH mutant *Yersinia*, there is a significant inhibition in the ability of antigen specific T cells to respond to antigen stimulation. Our results provide evidence that *Y. pseudotuberculosis* can disrupt antigen receptor signaling in lymphocytes and prevent their subsequent activation and function. A consequence of this action could be a direct inhibition of the development of a specific immune response toward *Yersinia*.

CASPASE-1 KNOCK-OUT MICE ARE RESISTANT TO ORAL INFECTION BY *S. TYPHIMURIUM*

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S. typhimurium causes a systemic, typhoid-like infection in mice and induces apoptosis in macrophages. Recently, we have shown that macrophages lacking caspase-1 are not susceptible to *Salmonella*-induced apoptosis. Caspase-1 knock-out and wild type mice were infected gastrointestinally with wild type *S. typhimurium* and recoverable colony counts from infected tissues were compared. At 14 hours, cecal colonization was the same for both caspase-1-deficient and wild type mice. In contrast, more wild type mice were colonized in the Peyer's patches. At 24 and 48 hours, *Salmonella* had spread to the mesenteric lymph nodes, spleen and liver of wild type mice, whereas the mice deficient for caspase-1 were not colonized beyond the cecum. In ligated loop infections, *Salmonella* transcytosed the M cells overlying Peyer's patches in caspase-1 knock out mice. Therefore the lack of recoverable colony counts is likely due to the inability of *Salmonella* to induce apoptosis in caspase-1$^-$ cells in the Peyer's patch, suggesting apoptosis is required for establishing infection following oral inoculation. Supporting this model, is the result that equal numbers of *Salmonella* were recovered from the spleens of wild type and caspase-1$^-$ mice subsequent to intraperitoneal inoculation. We are currently characterizing the inflammation in infected tissues.

MICROBIAL DILEMMA IN SYMBIOSIS: TO SUPPRESS OR AVOID HOST DEFENSE?

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Compared to the encounters between plants and pathogens, the course of a symbiotic interaction is less contentious and leads to a close physical association of the two partners. A close look at the plant responses reveal that complex interactions are occurring and rejection of invading symbionts is high, and may occur at any stage of the infection process. Plants respond to structurally diverse oligosaccharides from microorganisms during symbiotic and pathogenic encounters but their roles are not fully elucidated.

Our strategy involved analyses of inducible defense reactions in soybean (*Glycine max*) that are activated in response to cyclic and linear β(1→3)(1→6)-glucans from symbiotic (*Bradyrhizobium japonicum*) and pathogenic (*Phytophthora sojae*) microorganisms. We isolated and characterized the cyclic β-(1→3)(1→6)-glucan synthesis locus from *B. japonicum* and identified two genes (*ndvBC*) which are required for their synthesis and symbiotic N-fixation. It was observed that cyclic β-(1→3)(1→6)-glucans of *B. japonicum* suppressed phytoalexin synthesis induced by a fungal β-(1→3)(1→6)-linear glucan elicitor in soybean cotyledons. *B. japonicum* glucans prevented binding of the elicitor to the putative membrane receptor protein, and inhibited Ca^{++} movements in cultured cells induced by the elicitor. This led us to hypothesize a suppresser role for the cyclic β-glucans in nodule development. This implies the existence of an endogenous elicitor in *B. japonicum* and we have evidence for the occurrence of such an elicitor. The elicitor from *B. japonicum* induced Ca^{++} movements in transgenic soybean cells which were suppressed by cyclic β-(1→3)(1→6)-glucans. Data will be presented indicating the involvement of the host in regulating synthesis of cyclic β-(1→3)(1→6)-glucans which appear to have a suppressor-type function during infection and nodule development.

Bhagwat *et al.*, Plant Physiology 119:1057-1064, 1999; Mithöfer *et al.*, Planta 207: 566-574, 1999. Supported in-part by USDA, NRI Competitive Grants Program.

LEARNING FROM SPIROCHETE GENOMES

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Little has been learned about Treponema pallidum, causative agent of syphilis, using conventional molecular genetic approaches since this organism cannot be continuously cultured outside of animals in the laboratory. The whole genome sequence has dramatically changed this. Although most metabolic pathways are missing in this simple (1041 genes) microbe, those that are present provide insights into the mechanism of latency, whereby T. pallidum can remain dormant for decades in its human host. Few virulence genes can be identified by database comparison, suggesting a different strategy for host interaction in this spirochete as compared to better-studied pathogens. Some of those that have been found suggest hemolytic or toxigenic activities not previously associated with T. pallidum. Analysis of the long-range structure of the genome suggests that there are treponeme-specific islands that may encode new virulence factors. Methods to compare this genome to a related spirochete, T. pertenue, causative agent of yaws, and Borrelia burgdorferi, causative agent of Lyme disease, provide further evidence for the relevance of these novel genes to these different spirochetoses. Based on these insights, new vaccines and diagnostics may be possible to control and even eradicate this disease.

A GENOMIC ANALYSIS OF CHROMOSOME STRUCTURE AND MATING POTENTIAL IN *CANDIDA ALBICANS*

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The sequencing of the genome of *Candida albicans* (Ca), carried on at the Stanford University Genome center by Nancy Federspiel, Stew Scherer, and Ron Davis, has reached the 5.5x coverage point. This sequence has been assembled into 1631 contigs of greater than 2 kb. We, at Minnesota, have ordered the fosmid library of Ca into 45 contigs which have been placed on the eight chromosomes. The chromosome 7 fosmid contig map is complete. (1) We are completing the fosmid maps of the other chromosomes and placing the sequence contigs on these chromosomes. Comparison of the maps of clinical strains suggests a novel mechanism for karyotypic variation in this organism.

One of the genes identified by the sequencing project was the homologue of the *Saccharomyces cerevisiae* MATa1. The Ca mating type loci were identified and characterized by Christina Hull and Alexander Johnson at UCSF.(2) We have mapped these loci, called MTLa1 (MATa) and MTLalpha1 (MATa), to separate homologues of chromosome 5. We find that most clinical strains contain both mating loci although some strains have only one or the other MTL locus. We have used growth on sorbose as the sole carbon source, shown by Rustchenko (3) to promote loss of one homologue of chromosome 5, to isolate lab strains containing only MTLa1 or MTLalpha1. We are using marked strains of opposite mating type in an effort to induce mating behaviour in *Candida albicans.*

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SHIGA TOXIN PRODUCTION REQUIRES STX PHAGE INDUCTION

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The Shiga toxins (Stxs) produced by the many serotypes of Shiga toxin producing *E. coli* (STEC), such as *E. coli* O157:H7, are encoded in lysogenic lambdoid bacteriophages. These potent toxins are believed to result in the severe clinical sequelae, such as hemolytic uremic syndrome, that can result from infection with these enteric pathogens. Traditionally phages which encode virulence factors have been thought of simply as vectors facilitating the horizontal dissemination of virulence genes. How-ever, our studies suggest that the life-cycles of 'Stx phages' regulate toxin production and thereby influence pathogenesis. The genes encoding Stx2, *stx2AB*, are located in the 'late' region of the Stx phage genome 3' of the phage-encoded antiterminator Q and the late promoter p_R' and 5' of the phage lysis genes. To explore the importance of gene expression initiating at p_R' and Q-mediated antitermination, (which acts at a sequence over-lapping with pR'), in *stx2AB* expression, we constructed a deletion of p_R' in the genome of an Stx2-encoding prophage in an O157:H7 clinical isolate leaving intact *stx2AB* and its associated promoter. In vitro, compared to the wild type strain, the p_R' deletion strain made significantly less toxin even in the absence of the inducing agent mitomycin C. This suggests that the previously mapped *stxAB* promoter plays a minimal role in toxin production and that transcription which initiates at p_R' and requires Q-mediated antitermination is the major means of expressing *stxAB*. Since Q expression requires phage induction, these data also indicate that prophage induction is required for Stx production. We compared the amounts of Stx2 in fecal samples from mice inoculated with the wild type O157 strain or the strain harboring the p_R' deletion. In this in vivo model system, the amount of Stx found in the stools of the mice given the p_R' deletion strain was reduced 20-40 fold compared with the amount of toxin in the feces of the mice given the wild type strain. When these mice were treated with a phage inducing agent, the antibiotic ciprofloxacin, 1/6 of the mice inoculated with the pR' deletion strain mice died whereas 6/7 of the mice inoculated with the wild type strain died. Similar to our in vitro findings, these results suggest that in vivo phage induction, whether spontaneous or induced by intestinal factors or exogenous agents such as certain antibiotics, plays a critical role in Stx production. At least in the case of the Stx phages, the bacteriophage life-cycle is instrumental in the regulation of virulence gene expression.

PHAGE-MEDIATED TRANSFER OF VIRULENCE GENES IN SALMONELLA TYPHIMURIUM

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We have shown previously that *S. typhimurium* harbors two prophages — *Gifsy*-1 and *Gifsy*-2 — which contribute to bacterial virulence in mice. Both elements are inducible and once released as phage, they can lysogenize formerly cured recipient strains. Part of the contribution of the *Gifsy*-2 prophage to virulence results from its encoding a periplasmic [Cu,Zn]-superoxide dismutase (SodC) which improves the endurance of bacteria in the host, presumably by preventing oxidative damage. Both *Gifsy* prophages have been found, at corresponding locations, in all four *S. typhimurium* isolates tested thus far: strains LT2, ATCC14028, SL1344 and C52. More recently, we have identified three additional prophages which, unlike the above, are strain-specific. All three elements were isolated as phage from cultures of strains cured for both *Gifsy* prophages (hereafter referred to as the "doubly cured" strains). The Φssp-1 prophage is specific of the ATCC14028 background. Once induced, it can infect and lysogenize strains LT2 and SL1344. This phage carries *phoP/phoQ*-activated locus *pagJ* (described by S.I. Miller & coworkers) normally absent in strains LT2, SL1344 and C52. A second phage, Φssp-2, is released by the doubly cured derivative of strain SL1344. This phage contains the *sopE* gene identified by J.E. Galan & coworkers in strain SL1344, but otherwise absent in strains LT2, ATCC14028 and C52. Derivatives of strain LT2 lysogenic for phage Φssp-2 were obtained and found to have incorporated the *sopE* sequence. Finally, a third phage released by the doubly cured derivative of strain LT2 was identified as previously described *Fels*-1 phage. This phage contains the *nanH* gene (studied by E. Vimr & coworkers) normally not present in strains ATCC14028, SL1344 and C52. Derivatives of strains ATCC14028 and SL1344 lysogenic for the *Fels*-1 phage were isolated and found to carry the *nanH* gene. The heterogeneity of *Salmonella* prophage patterns might reflect strain adaptation to specific hosts and/or ecological niches.

IDENTIFICATION AND CHARACTERIZATION OF *GRVA SALMONELLA TYPHIMURIUM* ANTI-VIRULENCE GENE FOUND ON *GIFSY*-2 PHAGE

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We have identified a novel *S. typhimurium* virulence gene carried on the *Gifsy*-2 prophage. Contrary to classic virulence gene phenotypes, null mutants of *grvA* are more virulent than wild type when intraperitoneally inoculated in competition assays. Conversely, strains over-expressing *grvA* are less virulent in similar i.p. competition assays. When orally inoculated, these virulence phenotypes are present but are less dramatic than the i.p. phenotypes. Neither the *grvA* null mutant nor the *grvA* over-expressing strains have a growth advantage or disadvantage when grown in lab media. These results suggest that in a wild type situation, *grvA* may be involved in decreasing the pathogenicity of *S. typhimurium* in its host. The gene, *grvA*, is found in the lambdoid tail genes of *Gifsy*-2, a phage which has been shown to contribute to *Salmonella* virulence. This gene is a single open reading frame, which runs in the opposite direction of the phage tail gene operon. The open reading frame of *grvA* shows no homology to any gene in the NCBI database and is about 5-7 kb from the periplasmic superoxide dismutase *sodCI*, also carried on *Gifsy*-2. Despite the proximity of *grvA* and *sodCI*, the *grvA* null mutant does not have an effect on *sodCI* expression or vice versa. Our data suggests that the contribution of *Gifsy*-2 to *Salmonella* virulence is a complicated sum of both positive virulence factors such as *sodCI* and negative virulence factors such as *grvA*.

THE PRESENCE OF A PATHOGENICITY ISLAND IN SEROTYPES OF *SALMONELLA ENTERICA* SUBSPECIES I CORRELATES WITH ADAPTATION TO WARM BLOODED HOSTS.

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Host adaptation to warm blooded vertebrates is found in only one phylogenetic group within the genus *Salmonella*, termed *S. enterica* subspecies I. Since other phylogenetic lineages, such as *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, VI and VII are mainly associated with cold blooded vertebrates it has been speculated that *S. enterica* subspecies I evolved from a reptile adapted predecessor. However, it is currently unknown which genetic changes contributed to the expansion in host range of *S. enterica* subspecies I to include warm-blooded animals. Here we describe the identification and initial characterization of a pathogenicity island, located in the *xseA-hisS* intergenic region at 52 minutes on the *S. enterica* serotype Typhimurium chromosome. This region is approximately 25 kb; 15 kb is specific to serotypes of *S. enterica* subspecies I, while the remaining 10 kb is additionally present in *S. enterica* subspecies II and *S. bongori*. At least two determinants encoded on this island, *shdA* and *sinH*, are required for colonization of murine Peyer's patch tissue. The *shdA* gene is also required for efficient shedding in fecal pellets. Enhanced shedding is likely to increase the transmissibility of serotypes of *S. enterica* subspecies I between warm hosts, thus facilitating circulation within the population.

ORIGIN AND FUNCTION OF LEGIONELLA GENES REQUIRED FOR INTRACELLULAR MULTIPLICATION

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Legionella pneumophila is a facultative intracellular pathogen that grows equally well within a specialized vacuole in host cells and on bacterial media. Intracellular growth in human alveolar macrophages results in killing of the macrophages and severe pneumonia. In nature, Legionella *sp.* are found as parasites or endosymbionts of free-living protozoa, suggesting that these bacteria evolved specific mechanisms to take advantage of an intracellular lifestyle. The ability of the bacteria to prevent phagosome-lysosome fusion is a key determinant of their ability to survive and multiply intracellularly. Twenty-four *icm* (or *dot*) genes are required for the ability to grow intracellularly in both mammalian and protozoan hosts but are dispensable for growth on bacterial media. Null mutations in several *icm/dot* genes result in an inability to prevent phagosome-lysosome fusion. We hypothesize that a subset of the *icm/dot* gene products functions as a transfer apparatus for delivering effectors that interfere with phagosome-lysosome fusion. This idea is supported by the fact that 14 of the *icm/dot* genes exhibit strong homology to the transfer genes of the IncI plasmids, colIb-P9 and R64. In addition, wild-type *L. pneumophila* can conjugate RSF1010 in an *icm/dot* dependent manner and the presence of RSF1010 decreases intracellular multiplication. Additional support for this model comes from very strong homologies between the *icmT*, *icmS*, and *icmK* genes and random sequences derived from the genome of *Coxiella burnetti*, an intracellular pathogen that is the causative agent of Q-fever. Both *icmT* and *icmK* are homologous to genes found in the IncI plasmids, however the *icmS* sequence is not. Although *L. pneumophila* and *C. burnetti* are closely related evolutionarily, they have quite different intracellular fates. In contrast to *L. pneumophila, C. burnetti* resides in a fused phagolysosome. We propose that both organisms incorporated an IncI conjugation system to export effectors into host cells. It seems likely that the molecules not shared among them and the IncI plasmids correspond to the effectors that determine the distinct intracellular fates of these two intracellular pathogens.

VIRULENCE MECHANISMS OF BACTERIAL SUPERANTIGENS.
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There is a family of bacterial exotoxins termed superantigens due to their ability to activate a large percentage of the T cell population. These toxins simultaneously bind major histocompatibility complex (MHC) class II and the T-cell receptor (TCR) molecules at regions that map outside of the peptide-binding groove. This interaction results in stimulation of a larger percentage of the T cell population than observed with conventional antigens. Superantigens have been implicated in toxic shock syndromes, gastrointestinal food poisoning, and Gram-positive sepsis. This family of toxins includes the streptococcal pyrogenic exotoxins (Spes) A and C; the streptococcal superantigen SSA; the staphylococcal enterotoxins (SEs) A, B, C1-3, D, E, G; and toxic shock syndrome toxin (TSST-1).

SpeA is produced by lysogens of Streptococcus pyogenes associated with both scarlet fever and streptococcal toxic shock syndrome (STSS). Recently a 2.6 Å structure of SpeA was reported (Papageorgiou et al, EMBO J. 1999). This structure demonstrates that SpeA contains a zinc-binding site and is capable of forming disulfide linked dimers. Currently, the biological significance of the SpeA dimer is being investigated. The zinc-binding sites of SEA is involved in the recognition of class II MHC molecules. There are at least six alleles of speA, and speA3 is associated with STSS isolates. When compared to SpeA1, SpeA3 is more mitogenic and has a 10 fold increased affinity for the class II DQ molecule. SpeA3 differs from SpeA1 in one residue that maps immediately next to the zinc-binding site. Thus, the increased affinity of SpeA3 for the MHC molecule might reflect an increased affinity of this allelic form for Zn.

DELIVERY OF PROTEIN TO THE CONVENTIONAL MHC CLASS I PATHWAY FOR ANTIGEN PROCESSING USING *ESCHERICHIA COLI* EXPRESSING LISTERIOLYSIN O

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Listeria monocytogenes is a facultative intracellular bacterial pathogen that grows within the cytosol of infected cells. Listeriolysin O (LLO) is an essential determinant of pathogenicity whose natural biological role is to mediate lysis of *L. monocytogenes*-containing phagosomes. We have developed a system using *Escherichia coli* expressing cytoplasmic recombinant LLO to efficiently deliver co-expressed target proteins to the cytosol of macrophages [Higgins et al. (1999), *Mol. Micro.*, 31(6): 1631-1641]. Subsequent to phagocytosis, the *E. coli* are killed and degraded within phagosomes causing the release of LLO and the target protein from the bacteria. LLO acts by forming large pores in the phagosomal membrane thus releasing the target protein into the cytosol. Using immunofluorescence and timelapse video microscopy, delivery of protein to the cytosol was shown to be rapid, occurring within minutes following phagocytosis. Furthermore, the *E. coli*/LLO system is very efficient for delivering protein to the MHC class I pathway for antigen processing and presentation. T-cell activation studies with antigen processing inhibitors and TAP1 (transporter associated with antigen processing) deficient macrophages indicate that delivered protein is processed by the conventional MHC class I pathway for antigen processing and presentation. Moreover, similar antigen presentation was demonstrated using formalin-killed *E. coli*. Using this system, potentially large amounts of any protein that can be expressed in *E. coli* can be delivered to the cytosol of macrophages for subsequent processing and presentation on MHC Class I molecules without protein purification. This system has potential applications for the delivery of protein to other cell types and the delivery of antigenic protein *in vivo*.

MEMBRANE LOCALIZATION OF EXOS IS REQUIRED FOR EFFICIENT CYTOSKELETAL REARRANGEMENT OF EUKARYOTIC CELLS

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ExoS, a Type-III secreted ADP-ribosyltransferase from *Pseudomonas aeruginosa*, is a bifunctional cytotoxin that interferes with Ras-mediated signal transduction, and rearranges the cytoskeleton of eukaryotic cells. We have previously shown that ExoS can be divided into two functional domains. The amino-terminal 234 amino acids of ExoS (C234) is responsible for the disruption of actin filaments and cytoskeleton rearrangement, which can be blocked and reversed by treatment of CHO cells with CNF-1, a toxin that activates members of the Rho family of small GTPases. To investigate the mechanism of cytoskeletal rearrangement by C234, the effects of intracellular expression of C234 and deletion peptides of C234 were analyzed. Immunofluoresence analysis of CHO cells expressing C234 indicated that in vivo, C234 was not cytosolic, but instead localized to the perinuclear region. Upon Western blot analysis of fractionated CHO cells, C234 was found in the membrane fraction. To elucidate the domain within C234 responsible for membrane localization, deletion proteins of C234 were expressed in CHO cells and analyzed by immunofluoresence and Western blot analysis for localization and their ability to round cells. Proteins lacking either the amino-terminal 10 or 35 amino acids of C234, regions previously shown to be involved in protein secretion by the bacterium, were membrane localized and rounded CHO cells. Deletion of the amino-terminal 78 amino acids of C234 reduced membrane localization and rounding ability, while removal of the amino-terminal 107 amino acids of C234 abrogated membrane localization, and cell rounding, with the protein found in the cytosol. Carboxy-terminal deletion peptides lacking either an internal deletion of amino acids 108-180, or the carboxy-terminal 127 amino acids of C234 localized to the perinuclear region but were reduced in rounding ability, indicating that membrane localization is required, but not sufficient for efficient cytoskeletal rearrangement. Taken together, these data indicate that two regions in C234 are required for cytoskeletal rearrangment in eukaryotic cells, an amino-terminal membrane localization domain, and a carboxy-terminal effector region.

VIRULENCE PROPERTIES OF A *MANNHEIMIA (PASTEURELLA) HAEMOLYTICA* LEUKOTOXIN MUTANT TESTED IN A CALF-CHALLENGE MODEL

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Mannheimia (Pasteurella) haemolytica is the primary bacterial agent involved in a severe fibrinous pneumonia of cattle, commonly known as shipping fever. *M. haemolytica* expresses and secretes an RTX toxin that has both cytolytic and apoptotic activities against ruminant leukocytes. The leukotoxin (LktA) is considered the primary virulence factor of *M. haemolytica* and the clinical pathophysiology of shipping fever has been attributed to its activities. High titers of neutralizing anti-leukotoxin antibodies are correlated with enhanced resistance to the natural infection, further validating leukotoxin's role in the disease.

To examine this role, and in an attempt to create a potential live *M. haemolytica* vaccine, we created a mutant strain that produces and secretes inactive leukotoxin, and then tested the mutant for its ability to cause disease. A non-polar mutation within the leukotoxin transacylase gene, *lktC*, was created by allelic exchange and Cre-*lox* recombination. Since the *lktC* protein is required to post-translationally activate the LktA structural protein, the resulting mutant strain secretes inactive LktA, or pro-leukotoxin. Thirty-two colostrum-deprived Holstein calves were challenged, by transthoracic inoculation, with 3×10^8 or 3×10^9 cfu of the wild-type or mutant strain. Surviving calves were sacrificed four days post-inoculation and necropsies, bacteriological and serological analyses were performed. The LD_{50} of the mutant was ten-fold greater than the wild-type but lung lesion scores were reduced only three-fold. Though the mutant is less pathogenic than its wild-type parent, it retains considerable virulence in this model system. This suggests that the leukotoxin is not the sole virulence factor in *M. haemolytica.*

A HOMOLOGUE OF PERTUSSIS TOXIN ENCODED ON THE *YERSINIA ENTEROCOLITICA* CHROMOSOME

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Yersinia enterocolitica is an enteric pathogen of humans and other animals, most frequently associated with gastrointestinal syndromes, including mesenteric lymphadenitis and terminal ileitis. In a signature-tagged mutagenesis screen we identified a putative membrane-stress-response locus that is essential for virulence (homologous to the phage shock protein locus, *psp*, of *E. coli*). Immediately adjacent to the *psp* locus we have identified two *Y. enterocolitica* chromosomal genes that appear to encode an AB-type toxin (*toxAB*). The *toxA* gene is predicted to encode a protein with approximately 30% amino acid identity to the catalytic subunit of pertussis toxin. This is the first description of a putative bacterial toxin with significant overall homology to pertussis toxin. A glutamic acid residue that is essential for the ADP-ribosyltransferase activity of pertussis toxin is conserved as are other important amino acids. The ToxA and ToxB proteins are predicted to have signal sequences for export to the periplasm. Analysis of a Φ(*toxA-lacZYA*) operon fusion indicates that the genes are expressed and that expression is induced upon entry into stationary phase, a regulatory pattern in common with other *Y. enterocolitica* virulence factors.

The *toxAB* genes were apparently acquired by horizontal transfer since only the flanking genes were identified at the corresponding locations on the chromosomes of the two other pathogenic *Yersinia* species, *Y. pseudotuberculosis* and *Y. pestis*. Furthermore, the nucleotide composition of *toxAB* is significantly different from that of other *Y. enterocolitica* genes. *Y. enterocolitica* is a heterogeneous species with specific serotypes more frequently isolated from patients in certain geographic areas. At least two variants of the toxin genes are present in different *Y. enterocolitica* serotypes. These variants are only approximately 60% identical. This difference is far too large to be explained by genetic drift, suggesting significantly different selective pressures on the toxin genes of the various serotypes, or separate acquisition events.

THE PATHOGENESIS OF SALMONELLA TYPHIMURIUM INFECTION.

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Salmonella typhimurium infection provides a model to investigate a facultative intracellular pathogen since it carries well-defined virulence genes found on a plasmid and large insertions of DNA, pathogenicity islands (PI), common to most Salmonella species but absent from commensal enteric species. The PI SpiI is associated with genes concerned with epithelial cell invasion, while PI SpiII appears to be associated with genes concerned with survival inside the infected host after initial epithelial cell penetration.

S. typhimurium initially invades the M cells of the Peyer's patches located in the terminal ileum. M cell entry is associated with membrane ruffling and destruction of the M cell. In cell culture, Salmonella entry of macrophages and epithelial cells is associated with membrane ruffling; in the case of macrophages, entry leads to apoptosis of a major fraction of the infected cells. Initial cell entry and apoptosis are dependent on sipB genes that reside in SpiI. Regulation of cell entry is dependent upon a cascade of environmental signals including oxygen concentration, pH, and osmolarity that interact with the regulatory gene hilA.

Subsequent intracellular events appear to require the transcription of additional gene products. Intracellular Salmonella are found in a membrane-bound vesicle. Initially, specific genetic sequences are transcribed by intracellular bacteria in response to the acidic pH of the vacuolar environment. The same level of acidity negatively regulates hilA and represses spiI gene expression. Intracellular persistence and subsequent replication require an acid pH. If acidification of the vacuole is inhibited, the intracellular bacteria cannot replicate and are rapidly killed by the host cell. The Salmonella-containing vacuole is a privileged niche that excludes a number of cellular elements that are part of the normal endocytic pathway. Yet, other host cell markers are found early and in high concentration in intracellular vacuoles containing Salmonella. It is possible that the intracellular bacteria direct themselves to an existing host cell trafficking pathway that is distinct from the usual phagosome-lysosomal fusion seen for other phagocytosed bacteria. A vacuole containing virulent Salmonella does not undergo fusion with lysosomes.

Genes of a second pathogenicity island, SpiII, are essential for intracellular replication. Expression of these genes is under the control of a two-component regulatory system encoded within SpiII. The sensor arm of this regulatory cascade, ssrA, is activated by a low pH and requires the binding of the OmpR protein in the promoter region. It is likely that the transcriptional activator of the SpiII two component regulatory system is activated by other environmental signals. Mutation in either ssrA, ssrB or ompR will negate intracellular replication. The viability of intracellular bacteria is diminished in the ompR mutant background.

Finally, other unique DNA insertions are specifically associated with the ability of Salmonella to persist within an infected animal. One such insertion contains the transcriptional activator mig14 which does not effect entry or intracellular replication but does influence the persistence of the bacteria in the spleen and liver of infected animals.

THE EFFECT OF GRAIN-FEEDING ON THE DISSEMINATION OF *ESCHERICHIA COLI* FROM CATTLE TO MAN.

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Escherichia coli O157:H7 is a pathogenic bacterium that causes acute illness in humans, but mature cattle are usually unaffected and can be carriers. Only small numbers of cattle have *E. coli* O157:H7, but it can readily pass from one animal to another. *E. coli* O157:H7 can enter the human food supply from cattle via fecal contamination of beef at slaughter, and poorly cooked hamburger is a major cause of *E. coli* O157:H7 infection. Attempts to correlate the incidence of *E. coli* O157:H7 in cattle with specific diets or feeding management practices have given few, if any, consistent findings. However, recent work indicated that cattle diets may be changed from grain to hay to decrease the risk of *E. coli* dissemination from cattle to humans. This approach is based on general metabolic properties of *E. coli* rather than specific characteristics of strain O157:H7. *E. coli* cannot degrade starch, but it can ferment maltose and maltodextrins that are released by starch-degrading bacteria. When cattle were fed large amounts of grain (corn), *E. coli* numbers in the colon of cattle were greater than 10^7 cells per g feces, but cattle fed hay had less than 10^4 cells per g feces. Grain-fed cattle had higher concentrations of colonic volatile fatty acids than cattle fed hay, and these acids triggered the extreme acid-resistance of *E. coli*. Extreme acid resistance is a characteristic that allows bacteria to survive the low pH of the gastric stomach and re-colonize the intestinal tract. Cattle fed 90% grain had more than 10^6 acid-resistant *E. coli* per g feces, but acid-resistant *E. coli* were not detected in cattle fed hay. In vitro experiments indicated that *E. coli* strains from cattle fed hay or grain, and *E. coli* O157:H7 did not become acid resistant until volatile fatty acids were added to the growth medium. This result indicated that acid-resistance was an inducible characteristic of *E. coli* in general rather than a dietary selection of particular strains. Grain is often a cheaper feed than hay, but the period of hay feeding needed to decrease *E. coli* numbers and acid-resistance was relatively short (approximately 5 days).

A ROLE FOR BIOFILM DEVELOPMENT IN *PSEUDOMONAS AERUGINOSA* PATHOGENESIS.

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The development of biofilms requires the transition of bacteria from an individual, planktonic (free-swimming) existence to a surface-attached lifestyle in a complex, highly structured community. Although this transition has been shown to occur in response to a variety of environmental factors, the underlying molecular determinants required to regulate this process have not been elucidated. We have isolated mutants of *P. aeruginosa* unable to form biofilms on an abiotic surface. These mutants are designated *sad* for *s*urface *a*ttachment *d*efective. The study of the *sad* mutants by phase-contrast microscopy has enabled us to begin to elucidate the early steps in this novel developmental pathway. Furthermore, our studies suggest that genetic loci identified in our simple screen for mutants defective in biofilm development on an abiotic surface (including functions required for type IV pili biogenesis) may also play a role in pathogenesis *in vivo*.

THE STRUCTURE OF *PSEUDOMONAS AERUGINOSA* BIOFILMS IN LUNGS OF CYSTIC FIBROSIS PATIENTS MAY BE DICTATED BY HOST IMMUNE RESPONSE

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The leading cause of morbidity and mortality in patients with cystic fibrosis (CF) is chronic lung infection with *Pseudomonas aeruginosa*. The infecting strains typically undergo mucoid conversion, so CF lungs are often colonized with both nonmucoid and mucoid variants. The mucoid phenotype among these strains reflects overproduction of a linear polysaccharide called alginate. The appearance of mucoid strains correlates with the formation of a bacterial biofilm in the lungs containing microcolonies, the development of anti-*P. aeruginosa* antibodies and inflammation. The mucoid organisms are intractable, leading to a generally poor prognosis for the patient. The biofilm mode of growth enables the bacteria to resist the bactericidal function of polymorphonuclear leukocytes (PMNs) that release toxic oxygen byproducts such as hydrogen peroxide. In a recent report (Mathee et al., 1999, Microbiology, (145)1349-1357.), we showed that PMNs or hydrogen peroxide can cause *P. aeruginosa* to undergo mucoid conversion as in the CF lung. To mimic the inflammatory environment of the CF lung, we grew *P. aeruginosa* PAO1, a typical nonmucoid strain, as a biofilm in a laboratory flow chamber. When exposed to low-levels of hydrogen peroxide or activated PMNs, these biofilms produced mucoid variants, whereas no mucoid variants were isolated in untreated controls. All of the mucoid variants tested had a *mucA22* mutation, which is a common allele seen in CF isolates. The current study was undertaken to compare the biofilm architecture in vitro between PAO1 and its isogenic mucoid variant PDO300 in the presence or absence of treatment with activated PMNs. The development of biofilms of *P. aeruginosa* growing in laboratory flow chambers was followed over time by scanning confocal laser microscopy. Both nonmucoid and mucoid variants formed biofilms. In the early stages of biofilm development, the mucoid PDO300 typically formed microcolonies whereas the nonmucoid PAO1 formed a confluent layer. In later stages, in the absence of stress induction, both the parent and mucoid variant developed similar biofilm architecture. Upon PMN treatment, the mucoid PDO300 continued to form a biofilm whereas the nonmucoid PAO1 failed to do so. When a mixed biofilm, coinoculated with a mixture of PAO1 and PDO300, was treated with activated PMNs, many microcolonies were observed in contrast to the untreated chamber. These microcolonies contain both nonmucoid and mucoid colonies, which appeared to mimic the mode of growth seen in vivo.

GROWTH OF *LEGIONELLA PNEUMOPHILA* IN *DICTYOSTELIUM*- A NEW MODEL SYSTEM FOR INTRACELLULAR PATHOGENESIS

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We discovered conditions in which the intracellular bacterial pathogen, *L. pneumophila*, grows inside of the single-celled slime mold *Dictyostelium*. During the infection bacterial titer increases over 150 fold in four days. Electron microscopy shows that the bacteria are growing inside of *Dictyostelium* in membrane-bound compartments, and characteristic of *L. pneumophila* infections, the replicative phagosome is associated with rough endoplasmic reticulum. *L. pneumophila dot/icm* mutants that are unable to grow in amoebae and macrophages are also unable to grow in *Dictyostelium*.

The ability of *L. pneumophila* to grow in *Dictyostelium* depends on the physiological state of the *Dictyostelium* cells. Cells grown as adherent monolayers in tissue culture wells are susceptible to infection and cytotoxicity by *L. pneumophila*. *Dictyostelium* grown in suspension in the same medium and temperature, are resistant to both cytotoxicity and infection.

We are utilizing *Dictyostelium* genetics to identify host cell functions required to support growth of *L. pneumophila*. We are testing known *Dictyostelium* mutants and REMI mutagenized *Dictyostelium* for resistance to infection by *L. pneumophila*.

DROSOPHILA-PSEUDOMONAS AERUGINOSA AS MODEL SYSTEM TO STUDY HOST-PATHOGEN INTERACTIONS

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Microbial pathogenesis is complex and multifactorial involving numerous components from both the pathogen and the host. We have developed an innovative model system to facilitate the study of the complex mechanisms that bacterial pathogens employ to attack the host and the host responses in limiting disease development. Using *Arabidopsis thaliana* infection model, we have demonstrated for the first time that a human isolate of *P. aeruginosa* (UCPP-PA14) can cause disease in both plants, nematodes and mice using a shared subset of virulence factors. In the light of the fact that the innate immunity pathway of *Drosophila melanogaster* and the interleukin-1 receptor (IL-1R)-NF-*k*B pathway in mammals are homologous, we predicted that Drosophila would be a suitable model system to study host-pathogen interactions. We compared the ability *of D. melanogaster* to resist simple septic injuries, infection by a non-pathogenic bacterium (*E. coli)* and *P. aeruginosa* (UCPP-PA14) by monitoring fly mortality rates. We found that flies quickly recover from simple septic injuries, and able to clear the infection *by E. coli* introduced during septic injuries. In contrast, PA14 is lethal towards *D. melanogaster* within 48 hours post infection with a LD_{50} of one bacterium. Thus, we successfully established that the insect *D. melanogaster* is susceptible to *P. aeruginosa* (UCPP-PA14) infections, and can be used as a model system to study complex host-pathogen interactions. This is the first "bona-fide" bacterial pathogen reported to infect *D. melanogaster*. To further validate our *Drosophila* pathogenesis model, we screened in flies a collection of **24** UCPP-PA14 transposon mutants that we have shown previously to exhibit reduced virulence in either *Arabidopsis*, or *C. elegans* and **12** biochemically-characterized mutants with defects in functions important for the persistence and severity of infections. At least **17** of these mutants exhibit reduced virulence in *D. melanogaster*. The majority of which are shown to be less virulent in our mouse model. Transcript analysis of the *D. melanogaster* innate immune responses during infection showed that flies infected *with P. aeruginosa* exhibited different pattern of induction as compared to the *E. coli*.

TWO DRUG RESISTANCE GENES FOR USE AS SELECTABLE MARKERS IN CLINICAL STRAINS OF CANDIDA ALBICANS. Janna Beckerman, Joshua Turner and P. T. Magee. University of Minnesota.

There is a variety of dominant selectable drug resistance markers available for fungal geneticists. However, the use of these markers in *Candida* has been notoriously unsuccessful. This is due to several reasons: 1) *Candida* species, and *C. albicans* in particular, have been found to be naturally resistant to most selectable markers available, including hygromycin, benomyl, cycloheximide, tunicamycin and blasticidin S (B. Magee, personal communication; R. Zamorano, personal communication) and 2), the fact that *Candida* species read the CUG codon as serine instead of leucine makes many corresponding resistance genes nonfunctional in *C. albicans*. Mycophenolic acid (MPA) resistance, conferred by a mutation of the IMH3 gene, which encodes inosine monophosphate (IMP) dehydrogenase, is a selectable marker that has been successfully used in *C. albicans*, for selection in spheroplast fusion[Scherer, et al. 1990Microbiol Rev. 54:226-41) and more recently, as a reporter for virulence gene activation *in vivo* (Staib et al. 1999 Mol Microbiol 32:533-46). Herein we report on the sequence and mutation conferring MPA resistance in a single copy integration, and use of this marker for oligo-mediated gene disruption. Although MPA resistance is a useful selectable marker, its usefulness is compromised due to the fact that *C. albicans* is a diploid, therefore requiring a second selectable marker in order to perform targeted gene disruption. We found that Bialophos (phosphinothricin, Glufosinate) was sufficiently toxic to *Candida* to permit selection for resistance. However, the commonly used BAR gene (phosphinothricin acetyltransferase) conferring resistance to bialophos did not function in *C. albicans*. We constructed a new BAR gene,with codon usage optimized for a high level of expression in *C. albicans* and lacking CUG codons. Preliminary evidence suggests that transformation of this gene into spheroplasts of *C. albicans* confers resistance to bialophos and thus is an dominant selectable marker.